AN ABSTRACT OF THE THESIS OF

Catharina Coenen for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on June 13, 1996. Title: Auxin and Cytokinin Interaction in Tomato (*Lycopersicon esculentum* Mill.).

Abstract approved: Redacted for Privacy

Terri L. Lomax

The phytohormones auxin and cytokinin control plant development through a complex network of interactions which include synergistic, additive, and opposite effects whose mechanisms are unknown. The auxin-insensitive *diageotropica* (*dgt*) mutant provided a tool to dissect the relationship between auxin- and cytokinin-induced responses in tomato. Morphological, physiological, and molecular data support the proposal that auxin and cytokinins control a common set of developmental processes through separate signal transduction pathways which interact downstream from the *DGT* gene product. Morphological traits of *dgt* plants, such as reduced root and shoot growth, reduced leaf complexity, and reduced apical dominance were phenocopied by exogenous cytokinin application to wild-type plants, demonstrating that cytokinins and the *DGT*-mediated auxin response control a common set of phenotypic characteristics. Because the *dgt* mutation had no detectable effects on the levels of endogenous cytokinins or on the cytokinin sensitivity of whole plants,

cytokinins were hypothesized to cause *dgt*-like effects on plant development through inhibiting auxin-induced responses. This hypothesis was supported by physiological experiments showing that auxin-induced elongation and ethylene synthesis were inhibited in cytokinin-treated wild-type and in untreated *dgt* hypocotyls. Differences between the effects of cytokinins and the *dgt* mutation on auxin responses became apparent at the molecular level. Experiments on the auxin-induced accumulation of transcripts for two ACC-synthase genes and one *SAUR* gene demonstrated that cytokinin treatment selectively reduced the auxin-induced expression of only one ACC-synthase gene, while the *dgt* mutation inhibited the auxin-inducibility of all three genes. The effects of the *dgt* mutation were thus more pleiotropic than the cytokinin effects, suggesting that cytokinins inhibit auxin-responses downstream from the *DGT* gene product. *In vitro* culture of *dgt* hypocotyl explants and calli demonstrated shared or interacting signal transduction pathways for auxin and cytokinin in the stimulation of cell division, and independent pathways for the control of organ regeneration and vascular differentiation.

Auxin and Cytokinin Interaction in Tomato (*Lycopersicon esculentum* Mill.)

by

Catharina Coenen

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed 13 June, 1996
Commencement June 1997

Doctor of Philosophy thesis of Catharina Coenen presented on June 13, 1996

APPROVED:

Redacted for Privacy

Major Professor, representing Botany and Plant Pathology

Redacted for Privacy

Chair of Department of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

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ACKNOWLEDGMENT

I thank my major professor, Terri Lomax, for her generosity, support, and enthusiasm, and for her faith in my abilities. I thank the members of my committee, Carol Rivin, Don Armstrong, Machteld Mok, Paul Farber, and Pat Breen for their interest in my work and my carreer, and for their committment to making time for me whenever I needed them. A special thank you to Paul for supporting my ventures into epistemology. I thank Don for his interest in teaching and the time he spent teaching me about teaching. My thinking about physiology and my writing skills have benefitted greatly from advice by Dave Rayle. I owe a big thank you to all my fellow lab members, past and present; special thanks to Rosie Hopkins, Peggy Rice, Glenn Hicks, Steve Verhey, Chris Gaiser, and Kyoung-Hee Kim for years of companionship through the ups and downs of laboratory research. Thank you to Andreas Nebenführ for patiently helping with every computer problem, and to Mary Ellard-Ivey for generous help with all my molecular endeavors. I thank Gretchen Spiro and Janet Hochfeld for years of supporting my health and sanity. I thank Maria Finckh, Laura Morrison, Tina Dreisbach, and Frank Gecina for sharing all the joys and sorrows of my years in Corvallis. I thank my grandmother, Charlotte Grossgebauer, for encouraging and inspiring me by surviving hard times with great courage and humor. I thank my sister, Stephanie Van der Meyden, for her friendship and love. I thank my parents, Doris and Hans-Gerd Coenen, for their help through long years of schooling, their unconditional love, and their willingness to let me find my own way. I thank Chris Lundberg for love, friendship, trust, and patience.

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I. INTRODUCTION

Plant hormones are small, organic molecules which are produced in specific organs or tissues, and which elicit defined responses either directly at the site where they are synthesized or after transport to other organs and tissues. Plant development is controlled by a tight network of interactions between several different classes of such hormones. Each class of hormones affects a large number of developmental processes, and, in turn, most developmental processes are controlled by more than one class of hormones. While a combination of biochemical, genetic and molecular approaches has led to progress in elucidating the mechanism of action of certain plant hormones, the interactions between the different hormones have received less attention. This study is aimed at identifying mechanisms through which the plant hormones auxin and cytokinin interact in controlling plant growth and development.

I.1 Auxin

The main auxin in most plants is indole-3-acetic acid (IAA). In some plant species, additional compounds, such as phenylacetic acid or 4-chloro-IAA, may contribute to endogenous auxin activity (Davies, 1995). Metabolic precursors of IAA (e.g. indoleacetaldehyde) or IAA-conjugates (e.g. indoleacetyl aspartate)

may also have auxin activity in certain tissues. In many physiological studies, synthetic auxins, for example 2,4-D (2,4-dichlorophenoxyacetic acid) or NAA (α-naphthaleneacetic acid), are applied instead of IAA because these compounds are less susceptible to degradation.

IAA is synthesized primarily in leaf primordia, young leaves, and developing seeds. Tryptophan or indole-3-acetamide serve as precursors for IAA synthesis (Bandurski et al., 1995). Transport of IAA from the shoot tips towards the roots is mediated by an auxin-specific basipetal transport process (Lomax et al., 1995) which is thought to result in a gradient of auxin from high concentrations in the shoot tips to low concentrations in the roots. The phloem is thought to provide an additional pathway for the rapid long-distance transport of auxin.

Physiological responses to auxin have been described for a wide range of plant systems. Generally, auxin stimulates cell enlargement, stem growth, cell division in the cambium as well as in tissue culture, vascular tissue differentiation, and root initiation. An asymmetric distribution of auxin mediates tropistic bending responses of stems, roots, coleoptiles, and pulvini to gravity and light (Kaufman et al., 1995). The basipetal auxin transport stream controls the maintenance of apical dominance (Bangerth, 1994), and basipetal auxin export from leaves delays leaf senescence and abscission (Davies, 1995). The movement of assimilates towards an auxin source is thought to be enhanced through an effect of auxin on phloem transport (Brenner and Cheikh, 1995).

Furthermore, auxin promotes flowering in Bromeliads (Metzger, 1995), is required for fruit set and growth in some species (Gianfanga, 1995), and delays fruit ripening (Ludford, 1995).

The mechanism of auxin action has been investigated in most detail in the elongation response of excised coleoptile, hypocotyl and stem segments (see reviews by Brummell and Hall, 1987; Cleland, 1995). Auxin-induced elongation is associated with two rapid responses: an increase in the transcript levels for specific genes (reviewed by Hagen, 1995; Abel and Theologis, 1996) and a stimulation of an enzyme at the plasma membrane, the H^+-ATPase (Felle et al., 1991; Rück et al., 1995; Cleland 1995). While it is clear that both gene expression and ATPase activation are required for cell elongation to occur, the exact biochemical mechanism by which these processes initiate auxin-induced cell enlargement is unknown. According to the acid growth theory (reviewed in Rayle and Cleland 1992; Kutschera, 1994), stimulation of the H^+-ATPase by auxin causes an increase in wall extensibility which leads to a rapid, turgor-driven expansion of the cells. Prolonged growth beyond this initial acid-growth response requires the activation of gene expression and the synthesis and secretion of cell wall materials.

In addition to stimulating H^+-ATPase activity in a variety of systems, auxin has also been shown to stimulate other ion fluxes across the plasma membrane of *Vicia* guard cells. The opening of voltage-gated anion-channels in response to high auxin concentrations (Marten et al., 1991) is not an auxin-specific response but can also be induced by a number of other anions (Hedrich and

Marten, 1993). In contrast to this unspecific response, physiologically active auxin-concentrations were shown to specifically modulate both inward- and outward-rectifying K^+-currents (Lohse and Hedrich, 1992; Blatt and Thiel, 1994). Although an association between auxin effects on the H^+-ATPase and K^+-fluxes appears likely, the sequence of and causal relationships between auxin-induced events at the plasma membrane have yet to be established.

A large number of genes have been shown to be influenced in their expression levels by auxin (reviewed by Guilfoyle, 1986; Theologis, 1986; Key, 1989; Hagen, 1995; Napier and Venis, 1995; Abel and Theologis, 1996). Genes which are rapidly (within 30 min) induced by auxin include: (1) the family of *IAA/AUX* genes, encoding small hydrophilic proteins (Abel and Theologis, 1996); (2) a family of genes encoding glutathione S-transferases (GSTs, van der Zaal et al., 1987; Takahashi et al., 1989; Takahashi et al., 1991; Van der Zaal et al., 1991; Takahashi and Nagata, 1992; Droog et al., 1993); (3) a number of genes encoding ACC synthase, the enzyme which catalyzes the first committed step in the ethylene biosynthesis pathway (Nakagawa et al., 1991; Botella et al., 1992; Yip et al., 1992; Zarembinski and Theologis, 1993; Abel et al., 1995); (4) the family of Small Auxin Upregulated RNAs (*SAUR*s) (McClure et al., 1987; Guilfoyle et al., 1993); and (5) the *GH3* gene (Hagen et al., 1991). The sequences of the *SAUR* genes and the *GH3* gene have not yet shown homologies to genes of known function. More recently cloned auxin-stimulated genes show homology to fatty acid desaturases (*ARG1*, Yamamoto, 1994), to a

late embryogenesis-abundant protein (*ARG2*, Yamamoto, 1994), and to a G-protein beta-subunit (*arcA*, Ishida et al, 1993).

The first auxin-inducible genes to be sequenced (Ainley et al., 1989) are now known as members of the large *IAA/AUX* family. Proteins encoded by members of this family have been shown to have extremely short half-lives and to possess nuclear localization signals (Oeller and Theologis, 1995), suggesting that they may function as transcriptional regulators. Presently, specific roles for these proteins in auxin-regulated processes have not yet been established.

A specific role for auxin-inducible GSTs in auxin-induced cell division, as proposed by Takahashi et al, (1989), is not supported by transgenic plants expressing antisense RNA for an auxin-inducible GST, which demonstrate that auxin-induced cell division proceeds even though GST transcript levels are greatly reduced (Boot et al., 1993). Many members of this family are also inducible by a number of other stimuli, such as salicylic acid (van der Zaal , 1987), or heavy metals (van der Zaal et al, 1987; Hagen et al, 1988; Takahashi et al, 1995), suggesting that they might play a role in general stress responses.

Auxin is assumed to exert its effects on membrane currents and gene expression through binding to membrane-bound or soluble receptor proteins. A number of different auxin-binding proteins have been described and cloned (recently reviewed in Jones, 1994; Venis and Napier, 1995). ABP1, a soluble auxin-binding protein, which was originally purified from maize coleoptiles (Löbler and Klämbt, 1985; Shimomura, 1986), binds active auxins and undergoes conformational changes in response to auxin binding (Napier and Venis, 1990;

Shimomura et al., 1986). Antibodies against this protein have been shown to block auxin responses at the plasma membrane of protoplasts in three different systems: hyperpolarization of tobacco protoplasts (Barbier-Brygoo et al., 1989), potassium currents in *Vicia* guard cells (Thiel et al., 1993) and activation of the H^+-ATPase in maize coleoptiles (Rück et al., 1995). In addition, anti-ABP1 antibodies inhibit the auxin-induced rise in phospholipase A_2-activity in isolated plasma membrane vesicles (Scherer and André, 1993). This proposed action of ABP1 at the plasma membrane is in contradiction to the finding that ABP1 is predominantly located within the ER lumen (Ray, 1977; Shimomura, et al., 1988; Jones et al., 1989) due to a C-terminal KDEL-motif (Yu and Lazarus, 1991) which functions as an ER retention signal.

Additional auxin-binding proteins have been cloned and identified as a superoxide dismutase (Feldwisch et al., 1994), a β-1,3-glucanase (Macdonald, et al., 1991), a soluble (Bilang et al., 1993) and a membrane-bound (Zettl et al., 1994) glutathione-S-transferase, and several β-glucosidases (Campos, 1992; Feldwisch, 1994). It is presently unclear whether these auxin-binding proteins can be regarded as auxin receptors which act in a regulatory network as proposed by Jones (1994), because physiological auxin concentrations have not yet been shown to regulate the activity of any one of these enzymes.

A number of second messengers have been proposed to convey the auxin signal (reviewed in Hobbie et al., 1994; Napier and Venis, 1995), but as yet no one convincing pathway for auxin-induced signal transduction has been

established. Whereas experiments using ^{31}P nuclear magnetic resonance (Talbott et al., 1988) failed to demonstrate auxin-induced changes in cytoplasmic pH, both auxin-induced alkalization (Tretyn et al., 1991; Thiel et al., 1993; Blatt and Thiel, 1994) and acidification (Felle et al., 1986; Felle, 1988; Gehring, et al., 1990; Irving et al., 1992) of the cytoplasm have been observed with optical imaging or electrophysiological techniques. These changes in intracellular pH are associated with changes in intracellular Ca^{2+} (Felle, 1988; Gehring, et al., 1990; Irving et al., 1992). Auxin-induced Ca^{2+}-release could be triggered by an auxin-induced inositide signalling pathway. Increases in inositol (1,4,5)-trisphosphate and inositol bisphosphate in response to auxin have been reported (Ettlinger and Lehle, 1988; Zbell and Walter-Back, 1988). However, there has been no further characterization or confirmation of this initial work. In addition, auxin specifically activates a phospholipase A_2 in isolated plasma membrane vesicles (Scherer and André, 1989; André and Scherer, 1991), suggesting that auxin signal transduction may be mediated by the release of lysophospholipids which activate a membrane-associated kinase (Martiny-Baron and Scherer, 1989).

The large number of cellular responses which occur within minutes of auxin application to cells or tissues necessitates the existence of multiple sites of auxin perception (as proposed by Jones, 1994), or of a highly branched auxin signal transduction network, or a combination of these two possibilities. A possible means to resolve this question is the combination of genetic (see section I.3) and biochemical approaches. It will be interesting to see, for

example, if the events elicited by auxin at the plasma membrane (e.g. changes in ion currents) and in the cytoplasm (e.g. changes in gene expression) are mediated through a single auxin receptor.

I.2 Cytokinins

Chemically, most natural cytokinins are derivatives of adenine that have a branched 5-carbon N^6 substituent. Cytokinin synthesis in higher plants (reviewed in McGaw et al., 1995) is thought to be initiated by the transfer of the isopentenyl group of Δ^2-isopentenyl pyrophosphate to the N^6 atom of adenosine monophosphate. The resulting N^6-isopentenyl-adenosine monophosphate is then modified by hydroxylation, dephosphorylation, reduction, deribosylation, or cis-trans isomerization to produce other active cytokinins such as zeatin, or dihydrozeatin, as well as their ribosides and ribotides. Cytokinin molecules can be inactivated either through oxidation or through N- or O-glycosylation. Although most rapidly dividing tissues likely synthesize some cytokinins, root tips and developing seeds are thought to be the important sources of cytokinins. From the root tips, cytokinins are transported to the shoots in the transpiration stream moving up through the xylem.

Biologically, cytokinins are defined by their ability to stimulate cell division and direct cell differentiation in tissue culture. It is thought that the stimulation of cell division by cytokinins plays a role in the growth of fruits and shoot tips (Davies, 1995). Cytokinins also promote shoot initiation in a number of tissue

culture systems and in crown gall tumors, and increased cytokinin levels lead to the release of lateral buds from apical dominance (Tamas, 1995; Cline, 1994). The induction of lateral root primordia and the formation of roots in tissue culture are inhibited by cytokinins. Cytokinins enhance stomatal opening in some species (Mansfield and McAinsh, 1995), and they promote cell enlargement in leaves, leading to an increase in leaf area (Letham, 1971). In many plant species, cytokinins can strongly delay leaf senescence (Richmond and Lang, 1957; Noodén et al., 1979; Smart et al., 1991; Singh and Letham, 1992; Gan and Amasino, 1996), and cytokinin application can also promote the conversion of etioplasts into chloroplasts (Davies, 1995).

The slow and complex nature of most cytokinin responses has made it difficult to identify a primary target for cytokinin action. While several proteins have been found to bind cytokinins, there is no evidence that any one of these proteins has a physiological function (reviewed in Napier and Venis, 1990). A number of studies have uncovered specific effects of cytokinins on gene expression (Andersen et al, 1996, and references therein; Dehio and de Bruijn, 1992), suggesting that cytokinins may exert at least part of their effects through influencing the transcript levels for specific genes. Recently, cytokinins have been found to activate a *cdc2*-like protein kinase involved in the stimulation of cell division in tobacco pith explants (John et al., 1993).

I.3 Auxin and Cytokinin Response Mutants

The use of mutants in a genetic, molecular, and biochemical exploration of plant hormone biology has been highly successful (reviewed in Reid, 1990; Hobbie and Estelle, 1994; Hobbie et al., 1994). A number of single-gene mutants in *Arabidopsis* have been selected for their resistance to auxin in root growth assays. These mutants show a variety of auxin-related phenotypic defects, confirming the proposed role of auxin in processes such as vascular differentiation, root branching, gravitropism, cell elongation, and apical dominance. The auxin-resistant *axr1* mutant has been cloned and the predicted gene product has sequence similarity to the ubiquitin activating enzyme E1 (Leyser et al, 1993). However, so far it is unclear whether the *AXR1* protein has a similar function to E1 and how its function relates to auxin responses. Another *Arabidopsis* mutant, *axr2*, is thought to be disrupted in an early step of the auxin response because *axr2*-tissues do not accumulate *SAUR* transcripts in response to auxin (Timpte et al., 1995). *SAURs* (small auxin up RNAs) constitute a family of gene transcripts with unknown function which are rapidly auxin-inducible and are expressed in most elongating plant tissues (McClure and Guilfoyle, 1987, 1989; Li et al., 1991).

A number of auxin-resistant mutants have also been isolated in tobacco (reviewed in Hobbie and Estelle, 1994). Similar to the *Arabidopsis* mutants, these mutants show a wide range of developmental abnormalities which confirm a role for auxin in many different developmental responses. Two of these

tobacco mutants were shown to be affected in the auxin-induced hyperpolarization of the plasma membrane (Ephritikhine et al., 1987; Stirnberg et al., 1995), which is now attributed to the activation of the plasma membrane H^+-ATPase by auxin (Rück et al., 1995). These studies demonstrate that these mutations affect an early step in the auxin response and suggest that the hyperpolarization response to auxin may be of physiological significance.

In addition to the auxin-resistant mutants in *Arabidopsis* and in tobacco, a well-characterized auxin-resistant mutant is available in tomato. The single-gene, recessive *diageotropica* (*dgt*) mutation pleiotropically affects plant development (Zobel, 1972). Mutant plants show altered leaf pigmentation and morphology, stem growth, and do not form lateral root primordia, unless the root apex has been severely damaged (Zobel, 1973). Mutant shoots respond normally to both gibberellin (Zobel, 1974, Scott, 1988) and ethylene (Zobel, 1973), and contain normal auxin levels (Fujino et al., 1988). The velocity of polar auxin transport is also unaffected by the mutation (Daniel et al., 1989, Muday et al., 1995). Reduced labeling of an auxin-binding protein in *dgt* shoots (Hicks et al., 1989) was later found not to be correlated with the *dgt* phenotype (Lomax et al., 1993).

Two rapidly auxin-inducible genes, *LeSAUR* and *LeAUX*, are not induced by auxin in *dgt* hypocotyl segments (Mito and Bennett, 1995), indicating that the mutation is likely to affect an early step in auxin-signal transduction. Hypocoytl segments (Kelly and Bradford, 1986) and roots (Jackson, 1979, Muday et al., 1995) of *dgt* show reduced sensitivity to auxin in elongation and ethylene

biosynthesis assays. The effect of the *dgt* mutation on the elongation of hypocotyl segments was found to be due to a lack of auxin-induced wall loosening (Daniel et al. 1989), thus demonstrating that auxin effects on wall loosening and on gene expression are likely mediated through a common signal transduction pathway requiring the *DGT* gene. The effects of the mutation were shown to be auxin-specific, because the mutant is not affected in its elongation and ethylene-synthesis response to the fungal toxin fusicoccin (Kelly and Bradford, 1986).

In contrast to the relatively large number of auxin-resistant mutants which were selected in several different species, only four cytokinin-resistant mutants have so far been reported in higher plants, one in tobacco and three in *Arabidopsis*. The tobacco mutant was found to be affected in general stress responses rather than being specifically resistant to cytokinin (Blonstein et al., 1991), and the *Arabidopsis* mutant *ckr1*, originally isolated in a screen for cytokinin-resistance of root growth, was later found to be allelic to the ethylene-insensitive *ein2* mutant (Cary et al., 1995). The two remaining *Arabidopsis* mutants were found to be specifically resistant to the inhibitory effects of cytokinin on root elongation. One of these mutants, *stp1*, is specifically affected in cytokinin-mediated root expansion (Baskin et al., 1995), whereas the other mutant, *cyr1*, pleiotropically affects shoot development (Deikman and Ulrich, 1995).

I.4 Interactions between Auxin and Cytokinins

The range of developmental responses which are controlled by auxin overlaps greatly with cytokinin-controlled responses. In some processes, such as the control of apical dominance (Tamas, 1995), the formation of lateral root primordia (Wightman et al., 1980; Hinchee and Rost, 1986), or the elongation of shoot segments (Vanderhoef and Stahl, 1975), the two hormones have opposing effects. In other responses, such as the stimulation of cell division in tissue culture (Skoog and Miller, 1957; Fosket and Torrey, 1969), or the stimulation of ethylene synthesis (Lau and Yang, 1973; Aharoni et al., 1979), auxin and cytokinin can act in the same direction, leading to synergistic effects.

Many different models could be invoked to explain the control of a common set of responses through two different hormones. The models depicted in Fig. I.1 are designed to give a simplified illustration of the different levels at which cytokinin could influence responses to auxin. In general, the two hormones could interact in a linear fashion if one hormone controlled the abundance of the other hormone (Fig. I.1.a), they could change each other's perception (Fig. I.1.b), signal transduction processes (Fig. I.1.c), or effects on gene expression (Fig. I.1.d), or they could interact at the response level (Fig. I.1.e). These models are not intended to imply that these are the only plausible scenarios. For example, there may be responses to auxin or cytokinin which do not involve changes in gene expression, hormone receptors may directly influence gene expression without an intermittent signal transduction chain, and

the depicted sequences of events may be complicated by feedback loops and interactions with other signalling networks.

A number of studies suggest that auxin and cytokinin may interact through regulating each other's abundance. For example, the conversion of exogenously-applied IAA to IAA aspartate in mungbean hypocotyls is inhibited by cytokinins (Yip and Yang, 1986), and the application of cytokinins increased the endogenous levels of free IAA in maize roots (Bourquin and Pilet, 1990). Conversely, experiments demonstrating that the basipetal flow of auxin from the apical bud controls the levels of active cytokinins in the internodes (Bangerth, 1994), suggest that auxin could act through controlling the concentrations of free cytokinins. A role for auxin in the control of cytokinin levels is further supported by recent work on callus and cell suspension cultures demonstrating that auxin enhances the inactivation of cytokinins via oxidative breakdown (Zhang et al., 1995) and via 7-glucosylation (Crouch and van Staden, 1995).

Experiments in which transgenic plants overproducing auxin or cytokinin were crossed, indicate that at least apical dominance may be regulated by the ratio between auxin and cytokinin rather than by the absolute concentrations of these hormones (Klee, 1994). Although these findings may invite the speculation that the two hormones competitively interact at a receptor, the mechanism by which plant tissues sense or respond to such ratios is not addressed through this type of work.

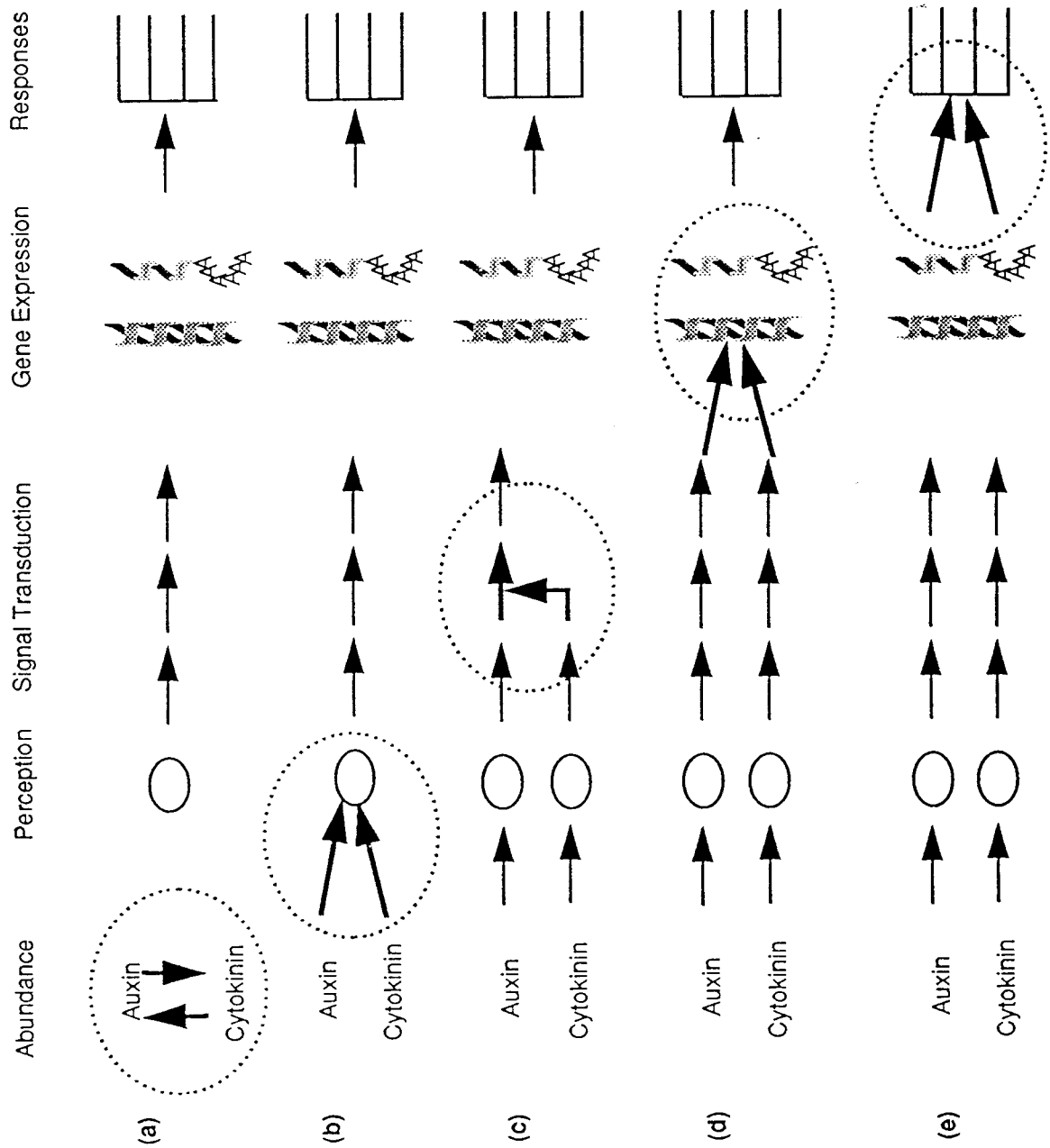

Fig. I.1. Diagram of possible auxin-cytokinin interactions.

There is little evidence for mechanisms through which auxin and cytokinin could modulate each other's perception or responses (Fig. I.1.b-d). Vanderhoef and Stahl (1975) proposed that cytokinins inhibit the auxin-induced elongation of hypocotyl segments by acting on the long-term elongation response while having no effect on the initial, fast, response to auxin which is "very similar to acid-activated growth". Recently, it has been found that auxin and cytokinin can also act in an opposing manner within single cells, as cytokinins were found to tighten transvacuolar actin strands in soybean root cells whereas auxins loosened these strands (Grabski and Schindler, 1996). Li et al. (1994) report a decrease of auxin sensitivity in transgenic plants with increased endogenous cytokinin concentrations, and there are reports of an inhibition of auxin-induced gene expression by cytokinin in tobacco suspension cells (Van der Zaal et al., 1987) and in tomato lateral root primordia (Young et al, 1994). Synergisms between auxin and cytokinin may also be mediated by interactions in signalling pathways upstream from gene expression, as cytokinin was found to change the induction kinetics of an auxin-inducible multiple-stimulus response gene through a process which may involve a kinase (Dominov et al., 1992).

I.5 A Mutant Approach to Auxin-Cytokinin Interactions

Hormone response mutants provide a powerful tool for the dissection of interactions between different signal transduction pathways. For example, the light-insensitive long hypocotyl (*hy*) mutants of *Arabidopsis* and the ethylene-

insensitive mutant *ein2* were used to demonstrate that the effects of cytokinin and light on hypocotyl elongation are mediated through separate signal transduction pathways (Su and Howell, 1995), and the ethylene-insensitive *ein1* and *ein2* mutants helped to show that the cytokinin-induced inhibition of root and hypocotyl growth is partly mediated by cytokinin-stimulated ethylene synthesis (Cary et al., 1995). Recently, cloning of the *HOOKLESS1* mutant led to the finding that ethylene regulates the expression of a gene which appears to control the perception or distribution of auxin (Lehman et al., 1996).

The cross-resistance which several of the auxin-resistant mutants show to other plant growth regulators confirms that the control of development by any group of hormones is closely linked to control processes by other hormones. Two possibilities to explain these cross resistances have been proposed by Klee and Estelle (1991), who postulate that either, cross-resistant mutants may define genes whose products transduce more than one hormone signal, or the first hormone may regulate the sensitivity to the second hormone. Beyond initial observations on the cross-resistance of some auxin-resistant mutants to cytokinin (reviewed in Hobbie and Estelle, 1994) and one report on endogenous cytokinin levels in an auxin response mutant (Pelese, 1989), mutants have not been used in a systematic investigation of auxin-cytokinin interactions.

The phenotypic similarities between *dgt* plants and cytokinin-overproducing transgenic tobacco plants open up the question whether auxin and cytokinin are linked in a linear signal transduction pathway in which one hormone controls either the abundance or the perception of the second hormone

(Fig. I.1a, b) or whether the two hormones interact further downstream. The experiments described here address these questions by characterizing cytokinin sensitivity and endogenous cytokinin levels in *dgt* seedlings and by comparing the effects of cytokinin and the *dgt* mutation on physiological and molecular auxin responses. Furthermore, the question of whether the effects of cytokinin on tissue growth and differentiation are dependent on a functional auxin response is addressed through a characterization of *dgt* hormone responses in tissue culture.

II. CYTOKININ AND THE *DIAGEOTROPICA* GENE AFFECT GROWTH AND DEVELOPMENT IN TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) THROUGH SEPARATE MECHANISMS

Catharina Coenen and Terri L. Lomax

Department of Botany and Plant Pathology and Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR

II.1 Summary

The hormones auxin and cytokinin have opposing effects on many aspects of plant morphogenesis. We compared the morphology of cytokinin-treated wild-type tomato plants with the phenotype of the auxin-insensitive *diageotropica* (*dgt*) mutant to determine the overlap in the range of morphogenic responses controlled by these two hormones. Wild-type plants grown in the presence of the cytokinin 6-benzylaminopurine closely resembled *dgt* plants in that they showed reduced root and shoot growth, decreased apical dominance, reduced leaf size and complexity, and increased chlorophyll and anthocyanin content. Traits of *dgt* which did not appear in 7-week old cytokinin-treated wild-type plants included longer hypocotyls and greatly shortened second internodes. Because of the striking similarities between cytokinin-treated wild-type plants and *dgt*, we tested whether the reduced sensitivity of the mutant to auxin may lead to or be a result of an increased sensitivity to cytokinin or to cytokinin-overproduction. With respect to inhibition of root and shoot growth, pigment accumulation, and ethylene biosynthesis, the cytokinin sensitivity of *dgt* was not increased compared to wild-type plants. Diferences in the endogenous cytokinin levels of wild-type and *dgt* tissues could not be detected in either etiolated or green seedlings. The auxin response which is mediated via the *DGT* gene thus does not appear to control cytokinin levels or sensitivity. Our results suggest that auxin and cytokinin modulate a common suite of morphological traits through

separate but interacting signal transduction pathways. The question of whether cytokinin acts by modulating auxin sensitivity is addressed in the following chapter (chapter III).

II.2 Introduction

The hormones auxin and cytokinin have long been known to interact extensively in regulating many different aspects of plant development as well as plant responses to the environment. We are interested in combining physiological, molecular and genetic approaches in unraveling the molecular mechanism(s) of this interaction. Research on auxin and cytokinin signal transduction has benefitted from an increasing number of auxin and cytokinin response mutants identified in a number of different plant species (Reid, 1990; Hobbie and Estelle, 1994). While some of these mutants appear to be affected specifically in their response to auxin (Hobbie and Estelle, 1995) or to cytokinin (Deikman and Ulrich, 1995), many of the *Arabidopsis* mutants isolated in screens for reduced sensitivity to one class of exogenous hormones in root elongation assays also show reduced responsiveness to at least one other class of plant hormones, reflecting the strongly interactive nature of hormonal signaling (Hobbie and Estelle, 1994). For example, the auxin-resistant *Arabidopsis* mutants *aux1*, *axr1*, and *axr3* show increased resistance to ethylene and cytokinin as well as to auxin (Hobbie and Estelle, 1994; Timpte et al., 1995), and the auxin-resistant *axr2* mutant was found to be resistant to ethylene and ABA

(Wilson et al., 1990). The *ckr* mutant of *Arabidopsis* which was originally isolated as a cytokinin-resistant mutant (Su and Howell, 1992) was later shown to be primarily affected in its response to ethylene (Cary et al., 1995).

The *dgt* mutant of tomato (*Lycopersicon esculentum* Mill.) is a single gene, recessive mutant with a pleiotropic phenotype (Zobel, 1972a). The plants have dark green, hyponastic leaves; thin, rigid stems; altered vascular development as well as primary and adventitious roots which lack lateral root primordia, unless the root apex has been severely damaged (Zobel, 1972b; Zobel, 1973), and elevated levels of anthocyanin (Lomax et al., 1993). Shoots of *dgt* are responsive to both gibberellin (Zobel, 1974; Scott, 1988) and ethylene (Zobel, 1973) but do not produce ethylene in response to auxin (Zobel, 1974). Hypocotyl segments (Kelly and Bradford, 1986) and roots (Jackson, 1979; Muday et al., 1995) of *dgt* show reduced sensitivity to auxin in elongation and ethylene biosynthesis assays. Auxin levels (Fujino et al., 1988) and the velocity of polar auxin transport (Daniel et al., 1989; Muday et al., 1995) in the mutant appear to be unaffected. Reduced labeling of an auxin-binding protein in *dgt* shoots (Hicks et al., 1989) was later found not to strictly correlate with the *dgt* phenotype (Lomax et al., 1993), and thus is unlikely to be the site of the *dgt* lesion.

Most morphological and physiological characteristics of the mutant are consistent with the hypothesis that the *DGT* gene product is involved in the perception or transduction of the auxin signal (Kelly and Bradford, 1986; Muday et al., 1995). Some of the traits exhibited by *dgt* plants, such as reduced root

formation, altered vascular differentiation, and reduced apical dominance, are also reported for tobacco plants which have reduced endogenous auxin levels due to the expression of a bacterial gene for IAA-lysine synthetase (Romano et al, 1991). However, plants transformed with the cytokinin biosynthesis gene *isopentenyl transferase* (*ipt*) from *Agrobacterium tumefaciens* also show *dgt*-like morphological changes such as reduced internode length, smaller leaves, a greatly reduced root system, reduced xylem content of the vascular system, and reduced apical dominance (reviewed in Klee and Estelle, 1991; Hamill, 1993; Klee, 1994).

Morphological changes induced by increased cytokinin concentrations, reduced auxin concentrations, or reduced auxin-sensitivity are thus very similar. There are three general possibilities which could explain these similarities: First, auxin could control plant morphology indirectly by influencing cytokinin metabolism or sensitivity of plant tissues. Second, cytokinin could control morphogenesis by altering auxin metabolism or perception. Third, separate signal transduction pathways for auxin and cytokinin could control a common set of morphological traits by interacting downstream from hormone perception. In this paper, we show that exogenous application of cytokinin to wild-type plants induces a phenocopy of the auxin-insensitive *dgt* mutant. By comparing cytokinin sensitivity and endogenous cytokinin levels in *dgt* and wild-type plants, we show that the auxin-response mediated by the *DGT* gene is unlikely to control plant development through controlling cytokinin sensitivity and endogenous cytokinin levels. In conjunction with investigations on the control of

auxin responses by cytokinin which we present in the following chapter (chapter III), our results suggest that cytokinin and the *DGT*-dependent auxin response alter a common set of developmental processes through separate signal transduction pathways which interact downstream from hormone perception.

II.3 Results And Discussion

II.3.1 Phenocopy of *dgt* Morphology with Exogenous Cytokinin

In order to test whether elevated cytokinin levels are able to induce a *dgt*-like phenotype in tomato, we grew wild-type plants in the presence of the cytokinin 6-benzylaminopurine (BAP), which is now known to occur as a natural cytokinin (Nandi et al., 1989). A comparison of morphology and pigment content of seven-week-old plants (prior to the onset of flowering) demonstrated that cytokinin treatment resulted in a phenocopy of morphological alterations which are exhibited by the auxin-insensitive *dgt* mutant. Wild-type tomato plants which had been grown in the presence of exogenous cytokinin showed stunting of root and shoot growth, reduced internode length, reduced leaf complexity, increased pigmentation, and reduced senescence of the cotyledons (Fig. II.1a, center) as compared with untreated wild-type plants (Fig. II.1a, left). For each of these traits, the altered morphology of cytokinin-treated plants closely resembled that of *dgt* control plants (Fig. II.1a, right).

Fig. II.1. Morphology of mature tomato plants. **(a.)** Seven week old plants: untreated wild-type (left), wild-type watered with 10μM BAP (center), and untreated *dgt* (right). **(b.)** Leaves from seven week old plants, arranged from cotyledon (left) to youngest leaf (right); untreated wild-type (top row), wild-type treated with 10μM BAP (middle row), and untreated *dgt* (bottom row). **(c.)** Leaves emerging from first internode of untreated *dgt* plant (left) and wild-type plant treated with 10μM BAP (right). **(d.)** Lateral branches emerging from first internode of a 3 month old *dgt* plant.

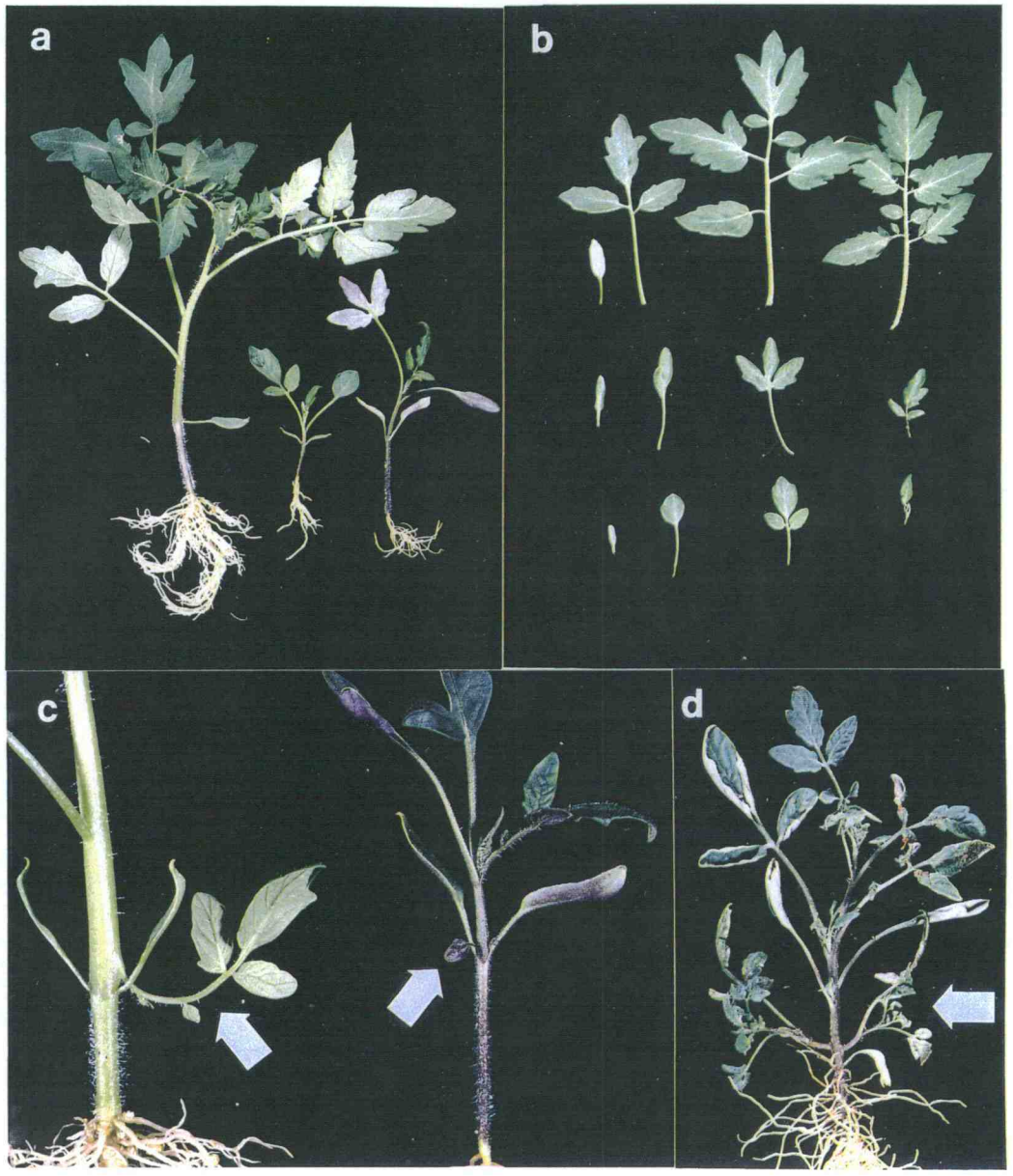

Figure II.1

Cytokinin treatment and the *dgt* mutation both reduced true leaf size, the number of leaflets per leaf, and the ornateness of the leaf margins in a strikingly similar manner (Fig. II.1b). Reduced leaf size and complexity also occurs in young tomato plants treated with the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) (Bedesem, 1958; Heinze, 1960), however, it is not known whether the TIBA treatment led to an increase or a decrease in the endogenous auxin concentrations in those studies. In potato plants transformed with the *iaaL* gene, a decrease in free IAA levels through conjugation of IAA into the less active IAA-lysine does not seem to affect the complexity of the leaves (Spena et al., 1991). While the role of IAA in determining leaf complexity is thus still largely unclear, exogenous cytokinin application convincingly mimicked the *dgt* leaf shape.

In 70% of the untreated *dgt* plants, downward-facing adventitious leaves (Fig. II.1c, right), which later developed into lateral branches (Fig. II.1d), grew out from the first internode. These leaves emerged directly above the cotyledonary leaf axils and showed an unusual angle of insertion. While lateral branches emerging from axillary buds of older tomato plants show an angle of insertion of about 30° relative to the main stem, lateral buds from the first internode of *dgt* grew out at about 120°, thus pointing downward. Growth in the presence of cytokinin resulted in the outgrowth of similar leaves with inverted insertion from the first internodes of wild-type plants (Fig. II.1c, left). In rare instances, untreated wild-type plants produced small, scale-like, structures in the same

location which always senesced soon after they developed and were absent at the conclusion of this experiment (data not shown).

Similar to elevated endogenous cytokinin levels in transgenic tobacco (Medford et al., 1989), exogenous BAP inhibited root development in tomato plants. The root systems of cytokinin-treated wild-type plants resembled those of untreated *dgt* plants (Fig. II.1a) in that they were severely stunted compared to wild-type roots. Stunting of the root systems of seven-week-old plants may reflect two components: reduced growth of the roots and reduced root branching. A role for cytokinin in the control of root branching in tomato is supported by the absence of root branching in cytokinin-overproducing tomato plants transformed with the *ipt* gene (Groot et al., 1995). Lack of root branching observed in *dgt* is caused by a block in the formation of lateral root primordia (Zobel, 1973). The root phenotype of the *dgt* mutant is thus convincingly phenocopied by application of cytokinin to wild-type plants.

II.3.2 Cytokinin Sensitivity is Not Altered in the *dgt* Mutant

The resemblance between cytokinin-treated wild-type plants and untreated *dgt* plants prompted us to investigate whether the *dgt* phenotype is caused by either an increased cytokinin-sensitivity or by elevated levels of endogenous cytokinins. We compared cytokinin responsiveness in *dgt* and wild-type in both mature plants and etiolated seedlings.

Leaves and stems of *dgt* are visibly more deeply pigmented than wild-type plants (Fig. II.1a) and this characteristic is also phenocopied by exogenous

cytokinin application. While darker leaf pigmentation was also reported for two auxin-resistant mutants of tobacco (Muller et al., 1985), it was not determined whether this was due to increased pigment concentrations in the leaves. We found that both chlorophyll and anthocyanin content of the first true leaf of *dgt* plants were approximately twice that of wild-type leaves (Fig. II.2a and II.2b). This increased pigmentation of *dgt* leaves was unlikely to result from an increased cytokinin sensitivity in the mutant because cytokinin treatment increased chlorophyll and anthocyanin contents in wild-type and *dgt* leaves in a similar manner (Fig. II.2c). As leaves of tobacco plants which are overproducing cytokinin were found to have doubled chlorophyll content (Li et al., 1994), it is possible that the increased pigmentation of *dgt* leaves indicates elevated endogenous cytokinin levels. Chlorophyll accumulation may also reflect a delay in the senescence of the first true leaf of *dgt*, since the increased retention of *dgt* cotyledons (Table II.1) demonstrates that the *dgt* mutation delays leaf senescence. The fact that delayed leaf senescence could also result from increased cytokinin levels is supported by observations on the effects of exogenous cytokinins on leaf sensecence (Richmond and Lang, 1957; Noodén et al., 1979), by correlations between leaf senescence and endogenous cytokinin levels (Singh et al., 1992), by several studies linking leaves cytokinin-overproduction in transgenic tobacco to delayed senescence of true leaves (Li et al., 1992; Smart et al., 1991; Gan and Amasino, 1996), and by our observation that delayed abscission of the cotyledons was copied by cytokinin-treatment in

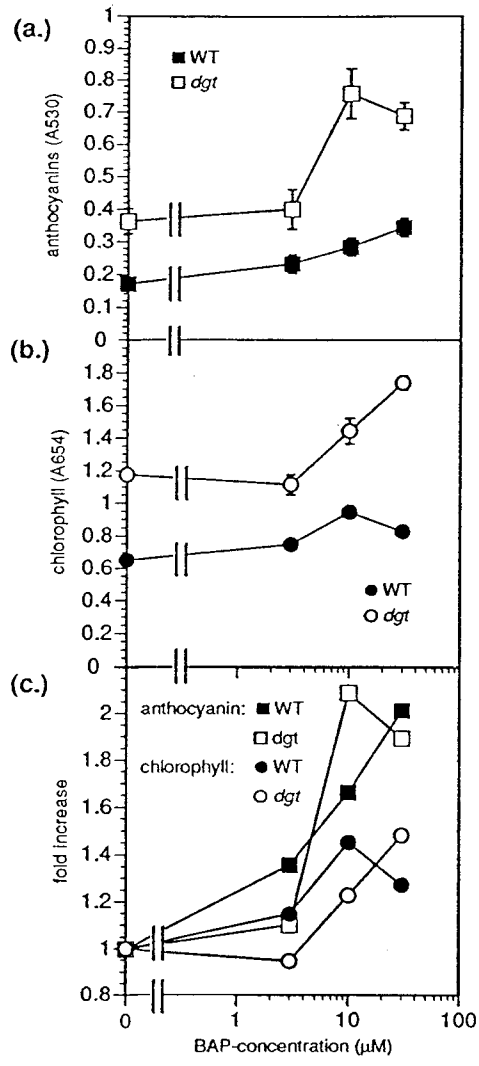

Fig. II.2. Increase in pigments extracted from the first true leaf of seven week old tomato plants grown in the presence of BAP. **(a.)** Anthocyanins. **(b.)** Chlorophyll. **(c.)** Increase in anthocyanins and chlorophyll content, normalized for absorbance of untreated controls. Error bars represent the SEMs for leaves from three plants. (squares = anthocyanins, circles = chlorophyll, open symbols = *dgt*, filled symbols = wild-type).

Table II.1: Morphological traits of seven week old wild-type (WT) and *dgt* tomato plants treated with or without BAP. Numbers in brackets represent SEMs.

	BAP (μM)	WT	*dgt*
number of plants harvested	0	18	24
	3	24	27
	10	19	16
	30	13	12
plants with cotyledons (%)	0	11.1	75.0
	3	87.5	100.0
	10	94.7	100.0
	30	100.0	100.0
plants with lateral branches (%)	0	0.0	70.8
	3	37.5	96.3
	10	15.8	56.3
	30	46.2	100.0
mean lateral branch fresh weight (mg)	0	0.24 (0.1)	38.9 (18.8)
	3	14.5 (5.9)	81.1 (39.3)
	10	13.1 (9.2)	14.1 (5.8)
	30	1.6 (1.0)	0.0 (0.0)

wild-type plants (Table II.1). While growth in the presence of 30 μM BAP induced anthocyanin levels in wild-type leaves which were similar to those of untreated *dgt* leaves, it needs to be borne in mind that anthocyanin accumulation in plants is regulated by a large variety of hormonal, environmental and developmental stimuli (McClure, 1975) and could thus be a secondary effect of a general stress condition, such as water stress caused by insufficient root and vascular development. In tomato, auxin inhibits the accumulation of the flavonoid rutin (Van Bragt et al., 1965), suggesting that auxin-insensitivity could also lead to increased anthocyanin accumulation.

The effects of varying cytokinin concentrations on the outgrowth of cotyledonary lateral buds and on cotyledon retention in wild-type and *dgt* plants are summarized in Table II.1. Cotyledon retention was increased by the *dgt* mutation and by BAP treatment of both wild-type and *dgt* plants at all concentrations tested (Table II.1). Apical dominance was greatly reduced by the *dgt* mutation with 70% of untreated *dgt* plants exhibiting outgrowth of lateral branches from the first internode as compared with 0% of untreated wild-type plants. While cytokinin treatment induced the outgrowth of lateral buds in wild-type plants, the relationship between cytokinin concentration and either the frequency of appearance or the fresh weight of the lateral buds was not linear in either genotype (Table II.1). The work with transgenic plants done by Klee and coworkers (summarized by Klee, 1994) suggests that apical dominance may be controlled by the ratio of auxin to cytokinin rather than by the absolute concentration of either hormone alone, and the results of Bangerth (1994)

suggest that auxin may control cytokinin levels in the internodes. Thus, either auxin-insensitivity or increased cytokinin production or sensitivity could cause the outgrowth of buds from the first internode in *dgt* (Fig. II.1c & Table II.1).

Stunting of shoot development in seven-week-old *dgt* and cytokinin-treated wild-type plants (Fig. II.1A) is reflected by a reduction in both shoot fresh weight (Fig. II.3), and internode length (Fig. II.4). Exogenous cytokinin reduced the shoot fresh weight of wild-type and *dgt* plants in a concentration-dependent manner (Fig. II.3), and with equal sensitivity (insert to Fig. II.3). Cytokinin treatment also reduced the length of both wild-type and *dgt* internodes (Fig. II.4). As observed for shoot fresh weight, sensitivity to cytokinin with respect to internode length was indistinguishable between *dgt* and wild-type plants. The effects of cytokinin and the *dgt* mutation on both internode elongation and shoot fresh weight accumulation appeared to be additive.

While internode lengths of wild-type plants treated with 10 μM BAP were in general very similar to those of untreated *dgt* plants (compare filled circles in Fig. II.4a with filled circles in Fig. II.4b), *dgt* plants show a characteristic extreme shortening of the second internode which was not observed in cytokinin-treated wild-type plants at any BAP concentration (Fig. II.4). Possible explanations for the particular susceptibility of the second internode to the effects of the *dgt* gene may lie in developmental changes occurring in the cytokinin to auxin ratio along the plant axis. These changes may not be accurately mimicked by exogenous application of cytokinin or may instead indicate a function of the *dgt* gene which does not involve regulation by cytokinin. The lack of elongation in the second

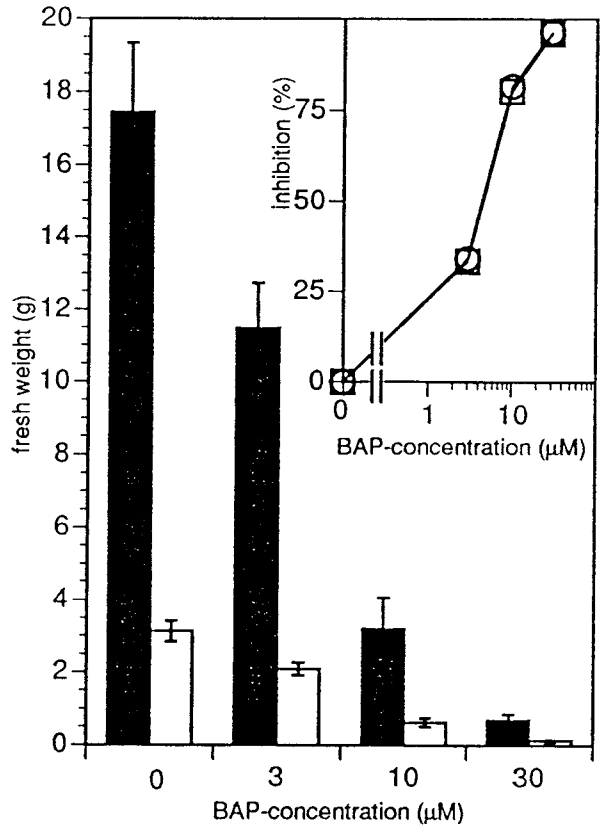

Fig. II.3. Inhibition of shoot fresh weight accumulation by BAP in seven week old wild-type (filled bars, squares) and *dgt* (open bars, circles) plants. Error bars show the SEM from all harvested plants (see Table 1 for number of plants in each treatment). Inset contains the same data expressed as % inhibition as compared with untreated controls.

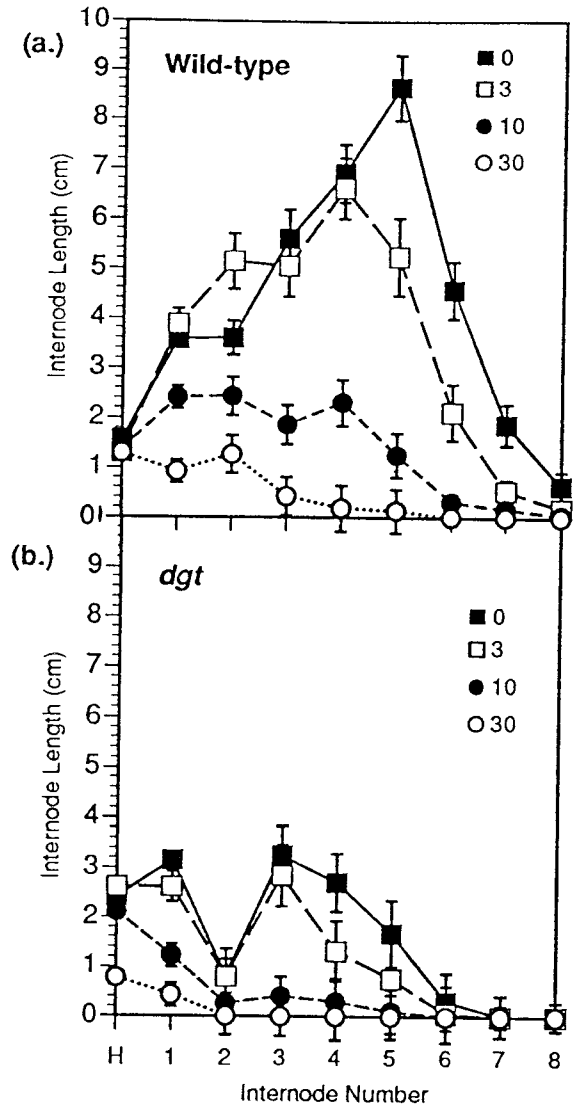

Fig. II.4. Reduction of internode length by BAP in seven week old wild-type **(a.)** and *dgt* **(b.)** plants watered with 30 (open circles), 10 (closed circles), 3 (open squares) and 0 (closed squares) μM BAP. Error bars represent SEMs (see Table 1 for number of plants in each treatment).

internode of *dgt* may also be related to the outgrowth of leaves from the first internode, reflecting a developmentally controlled shift of growth from the main shoot to a cotyledonary lateral bud.

Developmental changes in stem elongation were also suggested by comparisons of hypocotyl length in mature light-grown plants and in five-day-old etiolated seedlings. Similar to *amp1*, a cytokinin-overproducing mutant of *Arabidopsis thaliana* (Chaudhury et al. 1993), hypocotyls of five-day-old etiolated *dgt* seedlings were shorter than wild-type (28 vs. 36 mm). However, when the plants were grown in the light, 7-week-old *dgt* plants had longer hypocotyls than wild-type plants (24 vs. 16 mm). While the shorter hypocotyls of etiolated *dgt* seedlings were phenocopied by cytokinin-treatment in wild-type (Fig. II.5a), the longer hypocotyls typical for 7 week old *dgt* plants were not copied (Fig. II.4). Treatment of etiolated seedlings with various BAP concentrations reduced hypocotyl growth in both etiolated wild-type and *dgt* seedlings, indicating that both genotypes are equally sensitive to cytokinin with respect to the inhibition of hypocotyl elongation (Fig. II.5a).

Cytokinin inhibition of root growth in etiolated seedlings has been used extensively to quantify cytokinin sensitivity and to select cytokinin-resistant mutants in *Arabidopsis* (Su and Howell, 1992; Deikman and Ulrich, 1995). Our experiments on the cytokinin sensitivity of five-day-old etiolated tomato seedlings indicate that both the *dgt* mutation and treatment with exogenous cytokinin lead to reduced root growth (Fig. II.5b), because roots of untreated *dgt* seedlings grown on agar plates were shorter than wild-type roots and cytokinin inhibited

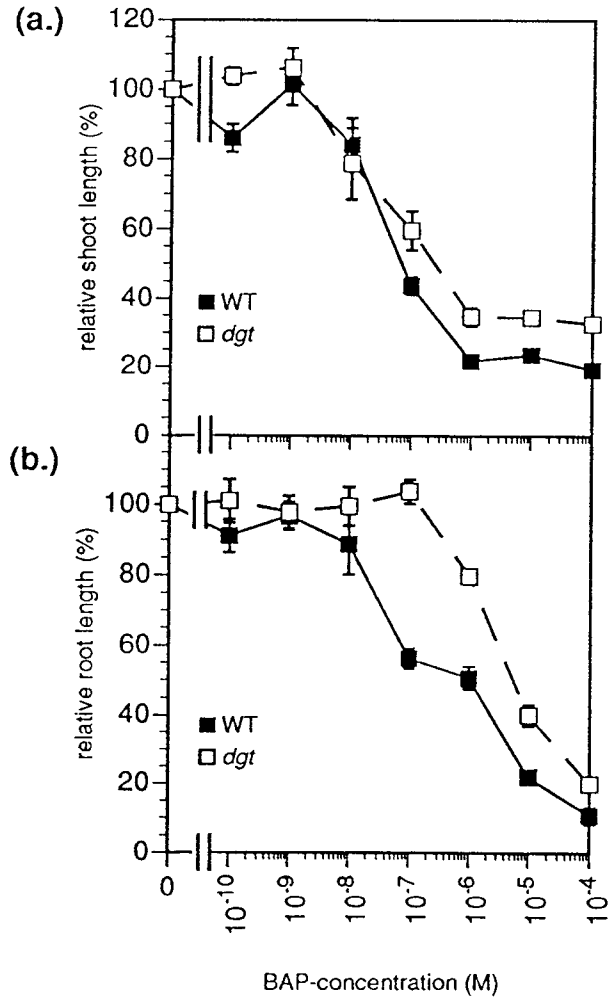

Fig. II.5. BAP-effect on length of **(a.)** hypocotyls and **(b.)** roots of five day old etiolated seedlings; wild-type (filled symbols) and *dgt* (open symbols). Error bars represent the SEMs from three independent experiments.

root growth further. Rather than being hypersensitive, *dgt* root growth appeared to be somewhat less sensitive to BAP (Fig. II.5b). As has been argued by Timpte et al. (1995) with respect to ethylene resistance in the *aux1* and *axr1* mutants of *Arabidopsis*, cross-resistance to cyokinin could either be a secondary effect of auxin resistance, or it may indicate that, in roots, the *DGT* gene also functions in the transduction of the cytokinin signal.

Many of the growth inhibitory effects of elevated cytokinin concentrations are now thought to be a result of cytokinin-stimulated ethylene synthesis (Cary et al., 1995). We used cytokinin-induced ethylene biosynthesis to further compare cytokinin sensitivity of *dgt* and wild-type seedlings. Our finding that the sensitivity of *dgt* seedlings to cytokinin was not increased in this response (Fig. II.6) agrees well with the results from cytokinin-induced growth inhibition. Furthermore, the functional ethylene synthesis response to cytokinin in whole seedlings of *dgt* is interesting in light of the inability of this mutant to synthesize ethylene in response to auxin (Zobel, 1974; Jackson, 1979; Kelly and Bradford, 1986). It confirms earlier results showing that *dgt* tissues can produce ethylene normally in response to stimuli other than auxin, such as anaerobiosis (Jackson, 1979; Bradford and Yang, 1980) and the fungal toxin fusicoccin (Kelly and Bradford, 1986), and demonstrates that cytokinin and auxin induce ethylene synthesis in whole seedlings by separate pathways.

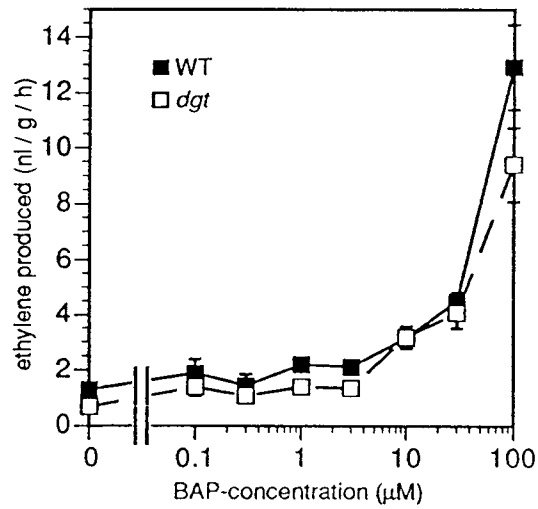

Fig. II.6. BAP-induced ethylene biosynthesis in etiolated wild-type (filled symbols) and *dgt* (open symbols) seedlings. Error bars represent the SEMs from three independent experiments with one to four replicates (*- n=3, ** - n=8, all others - n=11).

Taken together, data from studies measuring a variety of cytokinin responses in *dgt* indicate no alteration in cytokinin sensitivity. Mature wild-type and *dgt* plants appeared to be equally sensitive to cytokinin with respect to inhibition of pigment accumulation (Fig. II.2c), shoot fresh weight accumulation (Fig. II.3, insert), and internode elongation (Fig. II.4). Cytokinin response curves comparing the inhibition of hypocotyl elongation in *dgt* and in wild-type were also very similar (Fig. II.5a). In addition, ethylene biosynthesis was equally sensitive to BAP in *dgt* and wild-type seedlings (Fig. II.6). These experiments suggest that there is no increase in cytokinin sensitivity in etiolated seedlings of *dgt*.

II.3.3 Cytokinin Levels Are Not Altered in *dgt* Seedlings

A second possible explanation for the similarity between cytokinin-treated plants and the *dgt* mutant is that the *dgt* mutation results in increased levels of free cytokinin. We compared cytokinin levels in 3.5 week-old green tomato plants and in 5-day old etiolated seedlings. The etiolated seedlings were harvested for analysis at a point where growth inhibition in the mutant was clearly visible. Green plants were harvested when the first internodes were first starting to elongate so that growth inhibition in the mutant was just becoming apparent.

In etiolated seedlings there was no significant difference in either zeatin or zeatin riboside levels between mutant and wild-type tissue (Table II.2). In hypocotyl extracts of light-grown plants, the zeatin and zeatin riboside levels were variable, however, there was no indication that the levels for these

Table II.2: Endogenous cytokinin levels in untreated etiolated whole seedlings and hypocotyls of 3.5 week-old wild-type and *dgt* tomato plants. Results for etiolated seedlings represent three separate extractions, results for hypocotyls are from two separate extractions of two independently raised sets of plants. Numbers reported in this table are averages of triplicate ELISA assays performed on the HPLC fractions for each extract. (n.d. - not detected, a = experiment #1, b = experiment #2)

	extract	etiolated seedlings		light-grown hypocotyls	
	#	WT	*dgt*	WT	*dgt*
zeatin	1a	10.88	2.48	n.d.	n.d.
(pm/g fresh weight)	2a	12.29	20.63	32.66	n.d.
	3a	4.06	33.48		
	1b			110.95	n.d.
	2b			n.d.	n.d.
zeatin riboside	1a	11.34	20.25	301.51	286.74
(pm/g fresh weight)	2a	21.61	17.71	130.34	37.56
	3a	15.62	13.61		
	1b			362.29	n.d.
	2b			383.78	n.d.

cytokinins were elevated in the mutant. Zeatin or zeatin riboside were detected in only one out of four cotyledon and two out of five plumule samples, but their levels in *dgt* tissues were no greater than in wild-type tissues in any of these extracts (data not shown). Isopentenyl adenosine was detected in only two out of the four wild-type hypocotyl samples, and in none of the mutant samples. Neither isopentenyl adenine nor isopentenyl adenosine were detected in extracts from plumules and cotyledons of either genotype (data not shown). Thus, there was no indication that the auxin response mediated by the *DGT* gene results in increased cytokinin levels in either green or etiolated tissues. While several studies point to a control of cytokinin levels by exogenously-applied auxin (Palni et al., 1988; Bangerth, 1994; Crouch and van Staden, 1995; Zhang et al., 1995), and the levels of endogenous cytokinins were also found to be elevated in a NAA-tolerant mutant of tobacco (Pelese et al., 1989), our data suggest that either the *DGT* gene product is not involved in the control of cytokinin levels by auxin, or that the effect of the *dgt* mutation on physiologically active cytokinin pools cannot be detected with the methods at hand.

II.3.4 Conclusions

Most traits of the *dgt* mutant can be mimicked in wild-type plants by treatment with exogenous cytokinin. However, some traits of *dgt* (e.g. shortened second internodes and longer hypocotyls) were not copied. This may either be due to the limitations of the experimental system (exogenously-applied cytokinin may not lead to sufficiently high internal concentrations in wild-type tissues at the

correct times in development, or the cytokinin may not reach the responsive cells or compartments) or it may indicate that the *DGT* gene controls these morphogenetic processes independently from cytokinin.

We have tested a variety of responses to cytokinin in both the development of mature plants and in etiolated seedlings. As there was no indication of increased sensitivity to cytokinin in *dgt* in any of these responses, we conclude that the *dgt* mutation does not result in an increased sensitivity to cytokinin. Measurements of endogenous cytokinins in etiolated wild-type and mutant seedlings gave no indication of an altered cytokinin metabolism in *dgt* plants. The striking similarities in the effects of exogenous cytokinin and the *dgt* mutation on plant development are thus likely not due to either cytokinin hypersensitivity or overproduction in the mutant. The effects of cytokinin and the *dgt* mutation on morphogenesis appear to be for the most part additive, suggesting that auxin and cytokinin perception are not coupled in one linear signal transduction pathway but that their interactions occur further downstream. It remains possible that exogenous cytokinin alters either auxin sensitivity or signal transduction in wild-type plants and thus mimics the *dgt* phenotype. This possibility and a potential mechanism for cytokinin-auxin interaction is identified in experiments addressing the modulation of auxin responses by cytokinin (chapter III).

II.4 Materials And Methods

II.4.1 Plant Materials

For experiments testing the effect of exogenous cytokinins on wild type and *dgt* development, we used *dgt* and the isogenic parent VFN8. The more fertile Ailsa Craig (AC) background was used for studies on etiolated seedlings because of the large amounts of mutant seed required in these experiments. The morphological traits of *dgt* are the same in either the AC or VFN8 background (data not shown). Mutant seeds in the VFN8-background were originally a gift from Dr. K. Bradford, University of California, Davis, CA, USA. The *dgt* mutant extensively backcrossed into the AC-background was obtained from Dr. C.M. Rick, U.C. Davis. All seeds used in this study came from field plants propagated by selfing at the Oregon State University Botany Farm. Before sowing, seeds were surface-sterilized with 20% household bleach for 10 min, and rinsed extensively in tap water.

II.4.2 Morphological Measurements

Seeds were sown in Magenta boxes (7.5 cm x 7.5 cm x 10 cm, Sigma) on absorbent paper (Kimtowels) wetted with aqueous solutions of 0, 3, 10 and 30 μM benzyladenine (BAP). In preliminary experiments, it was determined that BAP concentrations of 1 μM or lower had little or no effect on any morphological characteristic, whereas 100 μM BAP was lethal (data not shown). After two days in the dark at 28°C the boxes were transferred to a light incubator equipped with

wide-spectrum fluorescent lights (GE Plant and Aquarium). Seedlings were grown for seven days at 28°C under a cycle of 16 h light (50 μE of PAR $m^{-2}s^{-1}$) and 8 h dark. Nine-day old seedlings were transplanted into 5 cm x 6 cm x 6 cm plastic pots containing a soil-free potting mix wetted with the appropriate BAP solutions. The mix consisted of 3 l vermiculite : 1 l expanded clay : 50 g Osmocote (14:14:14, N:P:K) : 3 g Micromax Micronutrients (Osmocote and micronutrients from Grace-Sierra Horticultural Products Company, Milpitas, CA). After 2 more days in the incubator, transplanted plants were grown in a greenhouse under natural light conditions at temperatures of 24°C (day) and 18 °C (night). During this period the plants were watered with the appropriate BAP solutions and fertilized as needed with Osmocote and micronutrients. Seven weeks after sowing, plants were scored for presence of cotyledons, photographed, and harvested for determination of pigment content, total shoot fresh weight, fresh weight of lateral branches, hypocotyl and internode length. The number of plants in each treatment is given in Table II.1.

II.4.3 Pigment Analysis

Pigments were extracted from the first true leaf of three plants for each treatment by chopping the leaf into pieces and extracting overnight on ice in 60 ml/g fresh weight of methanol containing 1 % hydrochloric acid. Extracts were stored at -20°C and their absorbance at 530 nm (anthocyanins) and 654 nm (chlorophyll) was measured in a Beckman DU-64 spectrophotometer.

II.4.4 Seedling Growth Measurements

For experiments on cytokinin-induced growth inhibition in intact seedlings, seeds were sown as described for auxin-responses and incubated for 2 days at 28°C in the dark. Germinated seeds with radicles of 3 to 5 mm length were placed on agar plates (15 cm diameter) containing 100 ml of 0.8% bacto-agar (Difco, Detroit, MI), MS-salts (pH adjusted to 6.5 with KOH) and the indicated concentrations of BAP. Sixteen seedlings were aligned on each plate with radicles pointing down and the plates were incubated vertically at 28°C in the dark. After three days, the root and hypocotyl length of each seedling was measured to the nearest mm with a ruler.

II.4.5 Ethylene Production

For experiments on cytokinin-induced ethylene biosynthesis in whole seedlings, ten seeds were sown in a 10 ml vial containing 1 ml of autoclaved agar-medium prepared as described for growth measurements. The open vials were incubated for five days in magenta boxes (7.5 cm x 7.5 cm x 10 cm, six vials to a box) at 28°C in the dark. For the last 8 hours of the fifth day, each vial was sealed with a serum stopper. One ml of the gas phase of each sample was analyzed on a Shimadzu gas chromatograph (Model GC-8A) equipped with a 4-foot Poropak Q-column and a flame-ionization detector. The results were normalized for the fresh weight of the seedlings in each vial.

II.4.6 Cytokinin Quantification

Seeds for wild type and mutant plants in the AC background were sown in plastic boxes (32 cm x 26 cm x 10 cm) onto two layers of Whatman filter paper #3 moistened with distilled water. Etiolated seedlings were grown in covered boxes for 5 days at 28°C in the dark. Seedlings were weighed, immediately frozen in liquid nitrogen, and stored at -80°C until analysis. Light-grown seedlings were raised by incubating the covered boxes for 9 days under a cycle of 16 h light (50 μE of PAR $m^{-2}s^{-1}$) and 8 h dark at 28°C. Seedlings were then transplanted and grown for two more weeks as described for morphological measurements. Plants were harvested when the first internode of the wild type seedlings was just beginning to elongate. Cotyledons, hypocotyls and plumules were collected on ice, rapidly frozen in liquid nitrogen and stored at -80°C until analysis.

Cytokinin analyses were performed as described in Banowetz (1992). Briefly, frozen tissue samples were ground in liquid nitrogen, extracted in methanol containing 100 mg of diethyldithiocarbamate as an antioxidant, diluted with 40 mM ammonium acetate (pH 6.5), and treated with wheat germ acid phosphatase. Extracts were purified by treatment with DEAE cellulose and passage over a C18 SepPak column (Waters Assoc., Bedford, MA, USA). Cytokinins were eluted from the SepPak column in methanol, taken to dryness and separated by HPLC on a 100 x 2.1 mm Hypersil ODS column (Hewlett Packard, Avondale, PA, USA). Cytokinins present in HPLC fractions were quantified by a fluorescence ELISA using two types of antibodies specific for

zeatin-riboside-like and isopentenyl-adenine-like cytokinins (described in Banowetz, 1992).

All chemicals were purchased from Sigma unless stated otherwise.

II.5 Acknowledgments

We thank Dr. Gary M. Banowetz at the USDA ARS in Corvallis, OR for conducting measurements of endogenous cytokinins, and Lisa H. Ouchida for technical assistance. This research was supported by NSF-grants to T.L.L.

III. CYTOKININS INHIBIT AUXIN-INDUCED ELONGATION AND ETHYLENE BIOSYNTHESIS DOWNSTREAM FROM AUXIN PERCEPTION AND THE *DIAGEOTROPICA* GENE PRODUCT

Catharina Coenen and Terri L. Lomax

Department of Botany and Plant Pathology and Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR 97331-2902, USA

III.1 Summary

Auxin and cytokinin regulate plant development through a complex set of interactions. In tomato (*Lycopersicon esculentum* Mill.), morphological traits of the auxin-resistant *diageotropica* (*dgt*) mutant can be phenocopied by growing wild-type plants in the presence of cytokinin (chapter II). Because this similarity is not due to increased cytokinin sensitivity or cytokinin overproduction in the *dgt* mutant, we investigated whether cytokinin acts by altering the sensitivity of plant tissues to the natural auxin indolyl-3-acetic acid (IAA). We compared the effects of cytokinin and the *dgt* mutation on auxin responses in tomato hypocotyl segments. Cytokinin did not cause a shift of the dose-response curves for auxin induction of elongation or ethylene synthesis, indicating that auxin sensitivity is not affected by cytokinin treatment. Instead, cytokinin treatment of wild-type hypocotyl segments reduced the amplitude of three auxin responses which are also affected by the *dgt* mutation: elongation, ethylene synthesis, and the accumulation of an mRNA from an auxin-inducible ACC synthase gene (*BTAS2*). The *dgt* mutation also affected the auxin-inducibility of a second ACC-synthase gene (*BTAS3*) and a tomato homolog of the *SAUR* genes; however, induction of these two messages by auxin was not reduced by cytokinin in wild-type tissue. Because the effects of the *dgt* mutation on auxin-induced gene expression were more pleiotropic than the effects of cytokinin, we propose that cytokinin inhibits the auxin response downstream from auxin perception and downstream from the *DGT* gene product. We conclude that cytokinin reduces the amplitude of auxin

responses at least in part through reducing the expression of a subset of auxin-inducible genes.

III.2 Introduction

The interactions of the plant hormones auxin and cytokinin throughout plant development are complex. While auxin and cytokinins have opposite effects in some developmental responses, they can also enhance each other's effects in other responses. The balance between auxin and cytokinins is thought to control the formation of roots and shoots in tissue culture (Skoog and Miller, 1957), as well as the emergence and distribution of lateral organs on the main plant axis, such as outgrowth of shoot axillary buds (Tamas, 1995) and lateral root formation (Wightman et al., 1980; Hinchee and Rost, 1986). An example of a synergistic interaction is the enhancing effect of cytokinins on auxin-induced ethylene production in pea stem sections (Fuchs and Lieberman, 1968) and tobacco leaf discs (Aharoni et al., 1979). In other cases, the auxin response is inhibited by cytokinins, as shown for the auxin-induced elongation of soybean hypocotyl segments (Vanderhoef et al., 1975).

Cytokinins could modulate auxin action at a number of different levels. First, cytokinins could control auxin abundance through controlling auxin metabolism or distribution. For example, cytokinins have been shown to inhibit the conjugation reaction by which exogenously-applied IAA is converted to IAA aspartate in mungbean hypocotyls (Yip and Yang, 1986) and to elevate the

endogenous levels of free IAA in maize roots (Bourquin and Pilet, 1990). The control of cytokinin metabolism by auxin has also been described (e.g. Crouch and Van Staden, 1995; Palni et al., 1988; Zhang et al., 1995). Second, cytokinins could alter the binding of auxin to a receptor mediating the auxin response. While a large number of auxin-binding proteins have been described (Jones, 1994; Napier and Venis, 1995), we are not aware of any evidence for an influence of cytokinin on auxin-binding to these proteins. Third, cytokinins could interrupt an auxin-induced signalling cascade, e.g. by inhibiting the activation of a protein kinase. In tobacco suspension cells, cytokinin likely enhances the auxin response of a multiple stimulus response (*msr*) gene through activating a protein kinase required for *msr* gene induction (Dominov et al., 1992). Alternatively, the interaction could take place during gene expression as has been described for nuclear hormone receptors in animals (Forman et al., 1995). Finally, cytokinins might inhibit the auxin response downstream from gene expression through changing the activity of an auxin-activated protein. An example of this is the interaction of cytokinin with an auxin-stimulated process in the initiation of cell division in tobacco pith explants. In this system, auxin induces the synthesis of a $p34^{cdc2}$-like protein but cytokinin is necessary for activation of the protein and for cell cycle activity (John et al., 1993).

Research on the interactions between plant signal transduction pathways has benefitted from the use of mutants which are disrupted in their response to hormones or environmental signals. For example, light-insensitive mutants of *Arabidopsis* were used to show that the effects of cytokinin on hypocotyl

elongation are independent from light effects (Su and Howell, 1995). Ethylene-insensitive *Arabidopsis* mutants helped to establish that cytokinin effects on hypocotyl and root growth are mediated by ethylene (Cary et al, 1995; Su and Howell, 1995). The de-etiolated mutants *det1* and *det2* are thought to define signalling intermediates which are shared between light- and cytokinin-induced signalling pathways (Chory et al., 1994).

To investigate the mechanism of interaction between auxin and cytokinin, we have used the *diageotropica* (*dgt*) mutant of tomato. Hypocotyl segments and roots of the *dgt* mutant show reduced sensitivity to auxin (Kelly and Bradford, 1987; Muday et al., 1995). This single gene, recessive mutant exhibits pleiotropic phenotypic effects, which include reduced apical dominance; stunting of root and shoot growth; dark green, hyponastic leaves; thin, rigid stems; and primary and adventitious roots which lack lateral root primordia, unless the root apex has been severely damaged (Zobel, 1972; Zobel, 1973). Mutant plants closely resemble wild-type tomato plants grown in the presence of cytokinin (chapter II). Because the *dgt* mutation does not increase the cytokinin sensitivity or the abundance of cytokinin in tomato tissues (chapter II), we hypothesized that the close resemblance between cytokinin-treated wild-type plants and untreated *dgt* plants may be caused by an inhibitory effect of cytokinin on auxin abundance, sensitivity, or responses.

In this paper, we investigate the mechanism through which cytokinin inhibits auxin action in tomato hypocotyl segments by comparing the influence of cytokinin and the *dgt* mutation on auxin-induced elongation, ethylene synthesis

and gene expression. The results suggest that cytokinin inhibits a subset of auxin responses in tomato hypocotyl segments without affecting the auxin-sensitivity of the tissues. We propose that cytokinin inhibits at least one auxin-induced signalling step downstream from auxin perception and from the *DGT* gene product but upstream from the activation of transcription by auxin.

III.3 Results

III.3.1 Auxin-induced Elongation and Ethylene Synthesis

To investigate the effect of cytokinin on the elongation of auxin-treated hypocotyl segments, we characterized segment elongation in the presence and absence of IAA and the cytokinin 6-benzylaminopurine (BAP) (Fig. III.1). Under our assay conditions, tomato hypocotyl segments elongated for 3 to 4 h, irrespective of hormone treatment. Auxin treatment (Fig. III.1, filled circles) increased the elongation rate during these first 3 to 4 h of treatment over the elongation rate of untreated control segments (Fig. III.1, filled squares). When cytokinin was supplied in addition to the auxin, the rate of auxin-induced elongation was reduced (Fig. III.1, open circles) while the elongation of control segments was not affected (Fig. III.1, open squares). Thus, rather than causing a delay in the auxin response, cytokinin treatment reduced the magnitude of the effect of auxin on the elongation rate of hypocotyl segments.

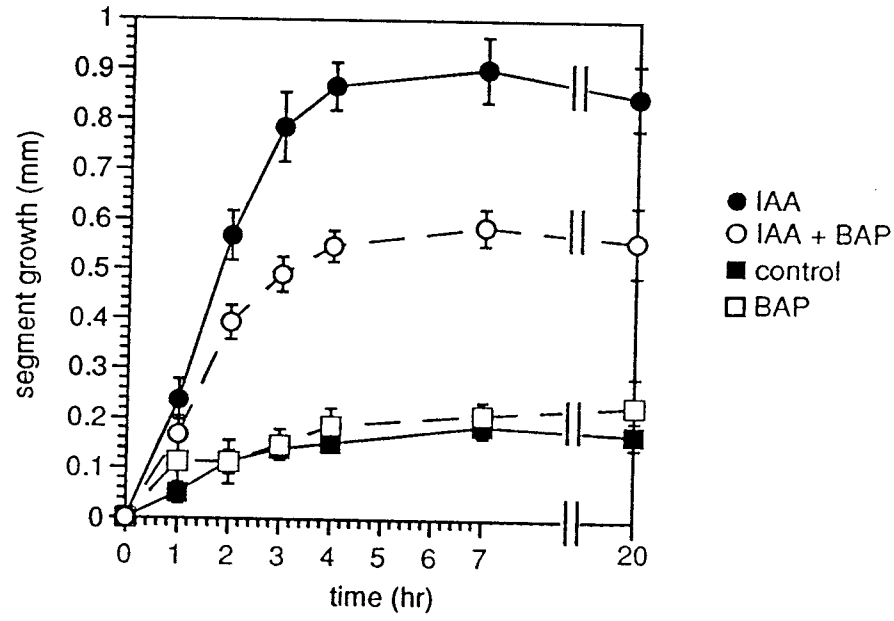

Fig. III.1. Time course of auxin-induced elongation in wild-type tomato hypocotyl segments in the presence and absence of 100 µM BAP. Circles represent treatments with 100 µM IAA, squares show elongation of control segments receiving no IAA. Open symbols represent segments receiving 100 µM BAP, closed symbols represent hypocotyl segments receiving no BAP. Error bars represent the SEM from three independent experiments.

The auxin-induced elongation of tomato hypocotyl segments was inhibited to a similar extent by BAP (Fig. III.2a, filled circles) and by the natural cytokinin zeatin (Fig. III.2a, open circles). None of the cytokinin concentrations tested had a significant effect on the elongation of segments in the absence of IAA (Fig. III.2a, squares). The inhibitory effect of cytokinin we observed in tomato segments was not as pronounced as reported for soybean hypocotyl segments (Vanderhoef et al., 1973), where cytokinin completely inhibited auxin-induced elongation. In addition, 10 to 100 μM BAP was required for maximum inhibition of auxin-induced elongation in tomato (Fig. III.2a, circles), whereas cytokinin concentrations as low as 4.2 μM result in maximum inhibition in soybean (Vanderhoef et al., 1973).

To investigate the effect of cytokinin on a second auxin response in hypocotyl segments, we characterized auxin-cytokinin interactions in the regulation of ethylene synthesis. While cytokinin concentrations of 0.1 to 10 μM did not inhibit auxin-induced ethylene biosynthesis, the auxin response was strongly inhibited in the presence of 100 μM BAP (Fig. III.2b, filled circles). Zeatin showed a similar pattern of enhancement at low concentrations, but did not consistently suppress the auxin effect at 100 μM (Fig. III.2b, open circles). Unlike studies with whole seedlings (Fuchs and Lieberman, 1968; Cary et al., 1995; chapter II), cytokinin did not by itself promote ethylene biosynthesis in hypocotyl segments (Fig. III.2b, squares; see also Fuchs and Lieberman, 1968). The effect of cytokinin on auxin-induced tomato segment elongation was likely not due to cytokinin-induced ethylene production because the concentration of

Fig. III.2. Concentration dependence of cytokinin inhibition of auxin responses in wild-type hypocotyl segments. Open symbols represent treatments with zeatin, closed symbols show treatments with BAP, circles represent segments treated with 100 µM IAA, squares represent control segments receiving no auxin. **(a.)** Auxin-induced elongation measured at 14 h after auxin addition. Error bars represent the SEM from three independent experiments. **(b.)** Auxin-induced ethylene formation measured during the period from 3 until 5 h after auxin-addition. Error bars represent the SEM from four (zeatin) or two (BAP) independent experiments.

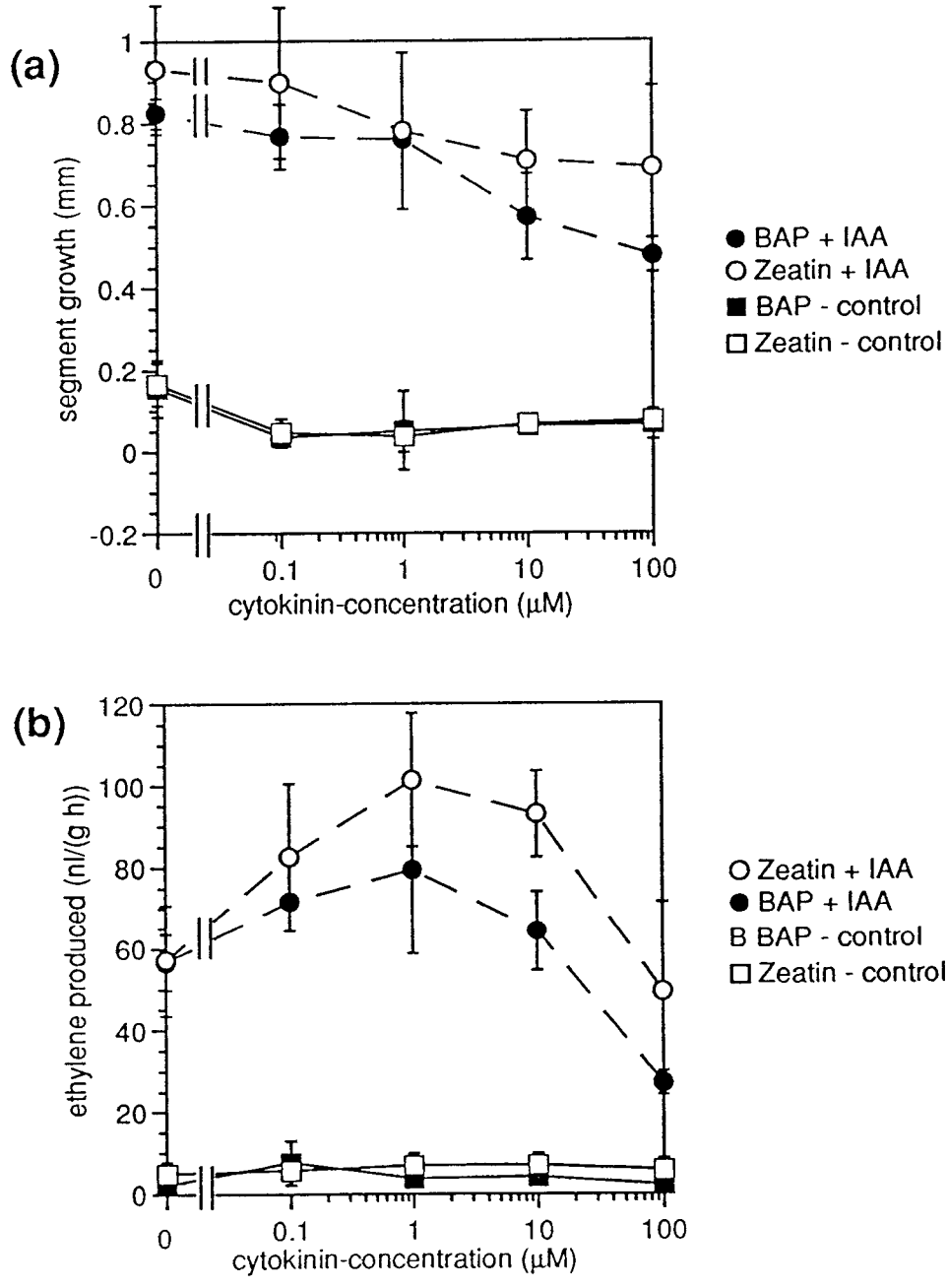
(a)
segment growth (mm)
1
0.8
0.6
0.4
0.2
0
-0.2
0
0.1
1
10
100
cytokinin-concentration (µM)
● BAP + IAA
○ Zeatin + IAA
■ BAP - control
□ Zeatin - control
(b)
ethylene produced (nl/(g h))
120
100
80
60
40
20
0
0
0.1
1
10
100
cytokinin-concentration (µM)
○ Zeatin + IAA
● BAP + IAA
B BAP - control
□ Zeatin - control

Figure III.2

cytokinin which was most effective in inhibiting elongation (i.e. 100 µM) also inhibited ethylene formation (compare Fig. III.2a and b).

To test the possibility that cytokinin caused a shift in the auxin sensitivity of hypocotyl segments, we compared auxin dose-response curves in the presence and absence of cytokinin (Fig. III.3). Auxin-induced elongation in wild-type segments was first observed at 1 µM IAA and did not show saturation within the range of auxin concentrations tested (Fig. III.3a, filled circles). This is in contrast to the results of Kelly and Bradford (1986), where the wild-type elongation response was first observed at 0.1 µM IAA and saturated at 1 µM. Differences between the elongation responses of wild-type segments in the two studies may have resulted from the use of different cultivars of tomato (we used the Ailsa Craig cultivar, whereas their study was done in the line VFN8), or incubation buffers (we used 1% sucrose, 5 mM MES at pH 6.0, they used a potassium phosphate buffer with potassium chloride, calcium and 3% sucrose at pH 5.2). In agreement with Kelly and Bradford (1986), we found that *dgt* hypocotyl segments did not elongate in response to up to 100 µM IAA (Fig.3a, closed squares). While we did not test the effect of IAA concentrations exceeding 100 µM, Kelly and Bradford (1986) observed a small stimulation of elongation in *dgt* hypocotyl segments at 500 µM auxin, indicating that the mutation causes a shift in auxin response curves to higher auxin concentrations.

Cytokinin reduced auxin-induced elongation of wild-type hypocotyl segments at all auxin concentrations which gave an enhanced growth rate (Fig. III.3a, compare filled and open circles). Our findings agree with the

Fig. III.3. Influence of cytokinin on the auxin sensitivity of hypocotyl segments. Circles represent wild-type segments, squares represent *dgt* segments, open symbols and closed symbols show treatments in the presence and absence of 100 µM BAP, respectively. **(a)** Auxin-induced elongation of hypocotyl segments. Note that the elongation of control segments receiving no auxin was subtracted. Error bars represent the SEM from independent experiments (n = 4 for wild-type, n = 3 for *dgt*). **(b)** Ethylene synthesis. Error bars represent the SEM from independent experiments (n = 4 for wild-type, n = 2 for *dgt*).

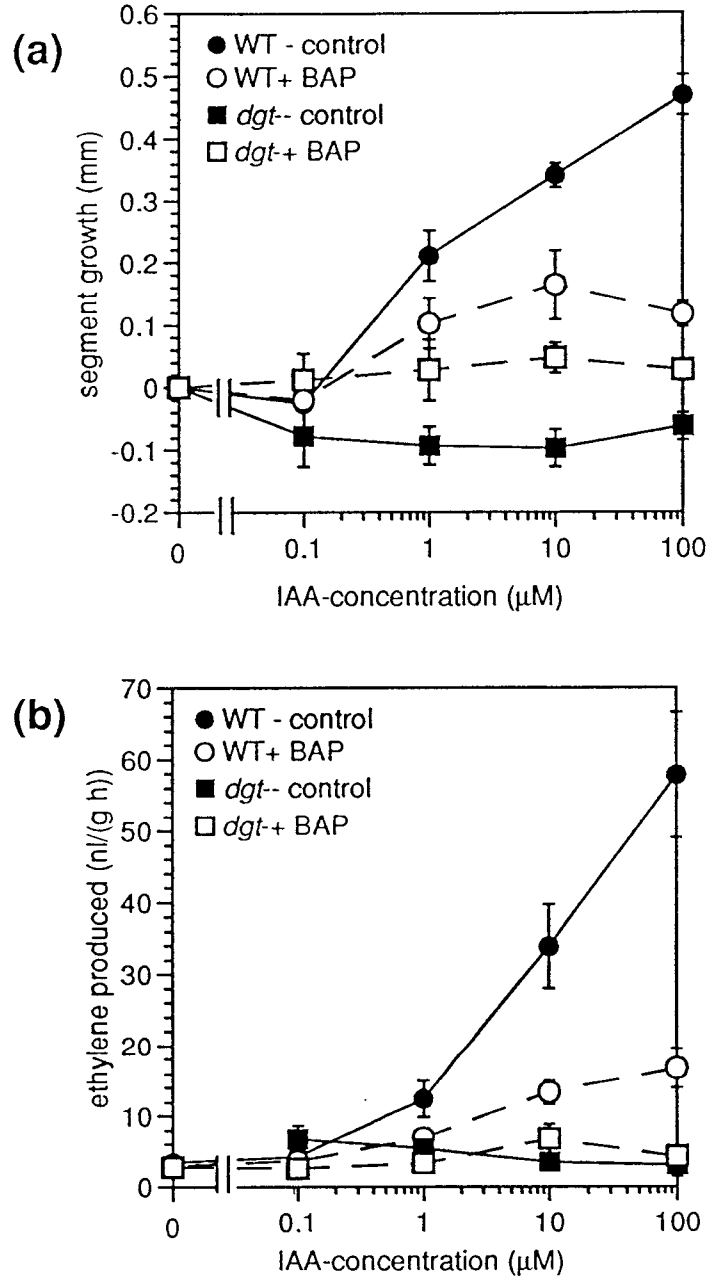
(a)
WT - control
WT+ BAP
dgt-- control
dgt-+ BAP
segment growth (mm)
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
0
0.1
1
10
100
IAA-concentration (µM)
(b)
WT - control
WT+ BAP
dgt-- control
dgt-+ BAP
ethylene produced (nl/(g h))
70
60
50
40
30
20
10
0
0
0.1
1
10
100
IAA-concentration (µM)

Figure III.3

conclusions of Vanderhoef et al. (1973) in that cytokinin effects on hypocotyl segment elongation cannot be overcome by increased auxin concentrations. The inhibitory effect of cytokinin relative to the auxin response appeared to increase with an increase in the auxin concentration (compare filled and open circles in Fig. III.3a). Because cytokinin caused a reduction in the magnitude of the elongation response rather than a change in the auxin concentration required to elicit this response, the effect of cytokinin on elongation appears to be different from the effect of the *dgt* mutation which has been shown to cause a shift in the auxin response (Kelly and Bradford, 1986; Muday et al., 1994). Cytokinin-treated wild-type segments and *dgt* segements showed normal responses to fusicoccin (data not shown). Thus, neither the *dgt* mutation nor cytokinin treatment of wild-type hypocotyls affected elongation processes in general; rather, they appeared to act specifically on auxin-mediated growth.

While cytokinin strongly stimulates ethylene synthesis in intact tomato seedlings (chapter II), cytokinin-treatment in the absence of auxin has no effect on ethylene synthesis in excised tomato hypocotyl segments (Fig. III.2b). The effect of cytokinin on the concentration dependence of auxin-induced ethylene synthesis in hypocotyl segments was very similar to the effect observed in the elongation response (compare Fig. III.3a and III.3b). Increasing auxin concentrations strongly stimulated ethylene synthesis in wild-type hypocotyl segments and cytokinin reduced the magnitude of this auxin response (Fig. III.3b, compare closed and open circles). Ethylene synthesis in mutant

segments was not influenced by either cytokinin treatment or by increasing auxin concentrations (Fig. III.3b, squares).

Although the data presented here cannot be interpreted kinetically in terms of a competitive or allosteric inhibition of auxin responses by cytokinin, these experiments indicate that the auxin concentration required to elicit a response in elongation (Fig. III.3a) and ethylene synthesis (Fig. III.3b) assays was not changed by cytokinin treatment. Rather, cytokinin appeared to reduce the amplitude of the responses elicited by auxin (Fig. III.3a and b).

III.3.2 Auxin-induced Gene Expression

Sustained elongation responses as well as ethylene synthesis in response to auxin treatment are mediated at least in part by an effect of auxin on gene expression. One type of transcript which accumulates prior to the onset of elongation responses are the *SAURs* (small auxin-upregulated RNAs; Hagen, 1995). Because the accumulation of *SAUR* transcripts is one of the earliest responses to auxin treatment, it provides a convenient means for testing how far upstream of the auxin response cytokinin acts. In agreement with previous reports (Mito and Bennett, 1995; Zurek et al, 1994), our northern blots showed that the auxin-induced accumulation of a tomato homolog for the soybean *SAUR* transcripts (*LeSAUR*) was strongly reduced in hypocotyl segments of the *dgt* mutant as compared with wild-type (Fig. III.4). In both wild-type and *dgt* plants, treatment with IAA and BAP together resulted in a larger signal than either hormone alone, suggesting that the effect of BAP on IAA-induced *LeSAUR*

accumulation is additive rather than inhibitory (Fig. III.4). The mechanism by which cytokinin inhibits auxin-induced elongation thus does not appear to involve the accumulation of *LeSAUR* message.

The cloning of three auxin-inducible ACC-synthase homologs (*BTAS1*, *BTAS2*, and *BTAS3*) by Yip et al. (1992) enabeled us to investigate the molecular basis for the interaction of auxin and cytokinin in the regulation of ethylene synthesis. We used RNase protection assays to quantify the relative steady state transcript levels for these genes in hormone-treated and untreated tissues. In accord with Yip et. al. (1992), we found that auxin induction of *BTAS1* was weak. Expression levels for this clone were variable and hormone-stimulated increases were small, so that it was unclear whether the *dgt* mutation had a significant effect on the hormone responsiveness of *BTAS1* (data not shown). Our findings support the view of Yip et al. (1992) that this gene might be responsible for the basal ethylene production observed in untreated hypocotyl segments and it will not be further discussed as an auxin-inducible gene here.

In contrast to *BTAS1*, the expression levels for *BTAS2* were clearly increased in response to auxin treatment in wild-type plants (Fig. III.5). Cytokinin by itself did not produce an appreciable effect on the message levels of *BTAS2*, but it strongly reduced the induction of the clone by auxin. The auxin induction of *BTAS2* message levels in *dgt* tissue was much smaller than in wild-type tissue and there was no induction of the gene by either BAP or a combination of BAP and IAA.

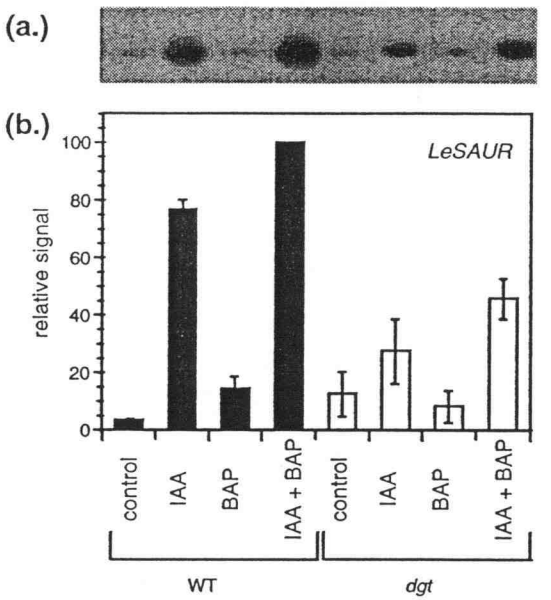

Fig. III.4. Influence of auxin, cytokinin and the *dgt* mutation on the expression of *LeSAUR* in etiolated hypocotyl segments as determined by northern blots. Hypocotyl segments were harvested and treated as described for elongation and ethylene biosynthesis assays. The final incubation was for 2 h in SM buffer containing the indicated hormones at 100 µM. **(a.)** Representative autoradiograph. **(b.)** Quantification of signals from autoradiographs of three independent experiments. Values from densitometer scans of films were expressed as percent of the highest signal in each respective experiment and subsequently averaged. Error bars indicate the SEM from three independent experiments.

Expression levels for a third ACC-synthase gene, *BTAS3*, were also dramatically increased by auxin treatment in wild-type tissue (Fig. III.5). Transcript levels for this gene were also increased by treatment with BAP, although to a much lesser extent than by auxin (Fig. III.5). The combined effect of IAA and BAP on this gene appeared to be additive. While we have been unable to detect *BTAS3* transcripts in untreated hypocotyl tissues of wild-type plants, there was a small signal in untreated *dgt* tissue (Fig. III.5). The level of this message in *dgt* hypocotyl segments was the same for all hormone treatments, showing no change in response to auxin, cytokinin or a combination thereof (Fig. III.5).

III.4 Discussion

We are interested in unraveling the mechanism through which exogenous cytokinin induces a phenocopy of many morphological traits of the auxin-insensitive *dgt* mutant. As the *dgt* mutation does not lead to increased cytokinin sensitivity or levels (chapter II), we asked whether cytokinin controls plant development through inhibiting auxin responses.

The molecular mechanisms underlying the interactions between auxin and cytokinin are unknown. As discussed in the Introduction, there are at least five different levels at which the two hormones could interact. First, auxin and cytokinin could control each other's abundance through regulating each other's metabolism or distribution. Second, auxin and cytokinin could compete, either

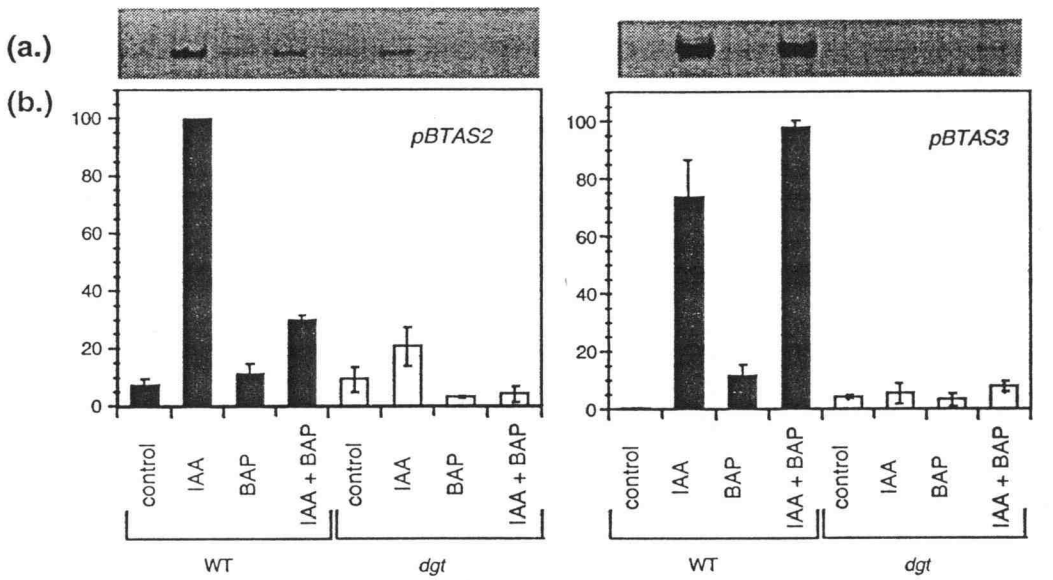

Fig. III.5. Influence of auxin, cytokinin and the *dgt* mutation on the expression of two ACC-synthase genes (BTAS2 and BTAS3) in etiolated hypocotyl segments as determined by RNase-protection assays. Treatments were performed as described for Fig. 4. **(a)** Fluorographs of representative polyacrylamide gels. **(b)** Quantification of signals from fluorographs. Values from densitometer scans of films from three independent experiments were expressed as percent of the highest signal and subsequently averaged. Error bars indicate SEM.

directly or indirectly, for binding to receptors controlling morphological functions. Third, cytokinin and auxin could modulate each other's signal transduction pathways, for example, a cytokinin-stimulated protein kinase or second messenger could reduce the activity of an auxin-stimulated kinase. A fourth possibility is that signal transduction intermediates from separate response chains for the two hormones interact to direct gene expression. Fifth, the products of auxin- and cytokinin-induced signal transduction might direct gene expression independently, and the resulting changes in the protein composition of the cell could determine the outcome of the response.

If cytokinin acts upstream from auxin perception by decreasing the abundance of auxin, or if it competes directly or indirectly for auxin binding to a receptor protein, the auxin response curves for cytokinin-treated tissues should be shifted to higher auxin concentrations, and the effect of cytokinin should be overcome by increasing the amount of auxin in the tissue. This type of interaction is found in other systems which are regulated by the ratio of auxin to cytokinin rather than by the absolute concentrations of the hormones, such as apical dominance (Klee, 1994; Cline, 1994) and organ formation in tissue culture (Krikorian, 1995).

We have characterized two physiological responses in tomato hypocotyl segments in which cytokinin inhibits auxin action: auxin-induced elongation (Fig. III.1, Fig. III.2a, Fig. III.3a) and ethylene synthesis (Fig. III.2b, Fig. III.3b). In both responses, the inhibitory effect of cytokinin did not decrease with increasing auxin concentrations (Fig. III.3), indicating that the effect of cytokinin cannot be

overcome by increasing the amount of auxin in the tissue. Our experiments also showed that cytokinin had no effect on the auxin-concentration at which the responses were first observed (Fig. III.3). Rather than changing the auxin sensitivity of the tissues, cytokinin reduced the amplitude of the auxin effect in both responses. Taken together, these data strongly suggest that cytokinin does not alter the levels of active auxin and inhibits auxin regulation of elongation and ethylene biosynthesis downstream from auxin perception.

A second criterion which can be used to evaluate different models for the inhibition of auxin responses by cytokinin is to see whether all responses to auxin are affected in a certain tissue. Cytokinin would be expected to inhibit all auxin-induced responses if it acted through reducing auxin abundance or through blocking auxin binding to a single receptor protein mediating all these responses. In contrast, cytokinin would be expected to influence only a subset of auxin-mediated responses if there were additional receptor proteins which were not influenced by cytokinin, or if cytokinin acted downstream from auxin perception on only one branch of a complex auxin-signal transduction pathway. A disruption occurring further upstream in a branched signal transduction pathway is expected to influence a larger spectrum of auxin responses than a disruption further downstream.

We have tested the possibility that cytokinin interferes with an early step in the auxin regulation of growth and ethylene biosynthesis by comparing the effects of cytokinin to those of a mutation in the *Diageotropica* gene. If cytokinin inhibited these auxin responses upstream from *DGT*, it would be expected that

cytokinin mimics all the effects of the *dgt* mutation. The influence of cytokinin and the *dgt* mutation were compared for five different auxin responses in tomato hypocotyl segments: elongation, ethylene synthesis, and the increase in message levels for three auxin-regulated genes. While all of these responses were affected by the *dgt* mutation (compare Figs. III.3, III.4, and III.5), cytokinin-treatment, affected elongation and ethylene formation (Fig. III.3), and the induction of one gene encoding an ACC-synthase homolog (*BTAS2*) (Fig. III.5), but it did not inhibit the auxin-induced increase in transcript levels for the *LeSAUR* (Fig.III.4) and *BTAS3* (Fig. III.5) genes. Other researchers have also reported an inhibitory effect of cytokinin on a subset of auxin-inducible mRNAs (Van der Zaal et al., 1987; Young et al, 1994), supporting the idea that cytokinin affects only part of the responses elicited by auxin. Our finding that cytokinin does not inhibit the expression of all the auxin-inducible genes whose expression is affected by the *dgt* mutation shows that cytokinin treatment does not lead to a complete phenocopy of *dgt* effects on the molecular level. Because the effects of the *dgt* mutation on auxin-induced gene expression are more pleiotropic than the effects of cytokinin, we propose that cytokinin inhibits the expression of a subset of auxin-inducible genes downstream from the *DGT* gene product.

The model in Fig. III.6 provides a summary of the effects of cytokinin and the *dgt* mutation on the steady-state levels of auxin-inducible RNA species. We have included *TR8* and *RSI1*, two genes whose expression in response to auxin and cytokinin was characterized in wild-type and *dgt* tomato roots by Young et al (1994). While the auxin-responsiveness of the *RSI1* gene is unaffected by

cytokinin and does not depend on a functional *DGT* gene, the four other auxin-inducible genes cannot be induced by auxin in mutant tissues. This suggests that *RSI1* is activated by auxin via a mechanism which is independent from the activation mechanism for the other genes (*LeSAUR*, *BTAS2*, *BTAS3*, and *TR8*). Cytokinin inhibits the auxin-inducibility of a subset of the *DGT*-dependent genes including the ACC-synthase gene *BTAS2* and the proteinase-inhibitor *TR8* (Young et al., 1994). The auxin-inducibility of the *LeSAUR* gene and the ACC-synthase gene *BTAS3* was not inhibited by cytokinin but depended on a functional *DGT* gene. The effects of the *dgt* mutation on the levels of auxin-inducible mRNAs were thus more pleiotropic than the effects of cytokinin, suggesting that cytokinin inhibits the induction of genes by auxin downstream from the action of the *DGT* gene product (Fig. III.6).

Further work is needed to investigate whether cytokinin has additional effects downstream from gene expression. For example, it is tempting to speculate that the observed inhibition of auxin-induced ethylene production in cytokinin-treated tissue might be caused by the influence of cytokinin on the transcription of *BTAS2*. However, our results do not permit us to exclude the possibility that cytokinin has additional effects on the auxin response downstream from ACC-synthase gene expression, such as inhibitory effects on ACC synthase and ACC oxidase enzymes. Similarly, two possible explanations remain for the the lack of reduction of auxin induction by cytokinin on the expression of the *LeSAUR* gene, which has been implicated in auxin-induced elongation. Induction of this gene by auxin might either not be essential for

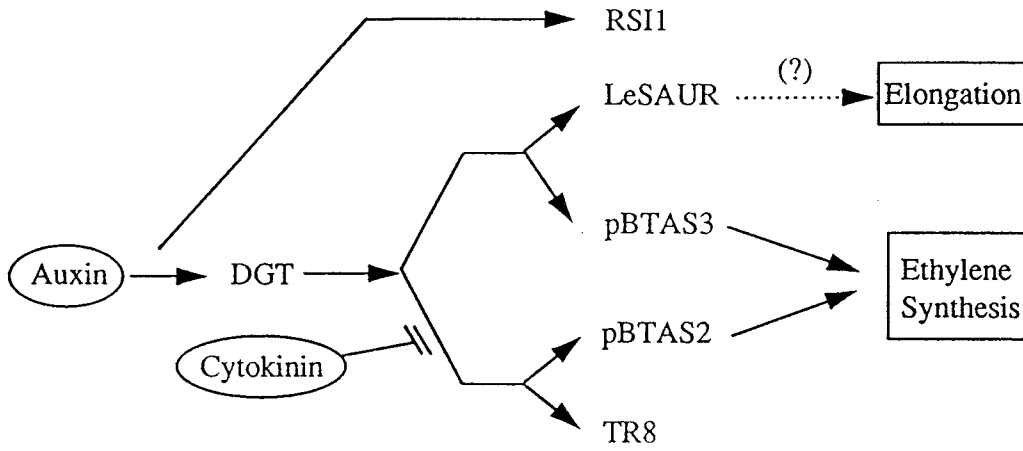

Fig. III.6. Model for the function of cytokinin and the *DGT* gene in the regulation of auxin-induced gene expression in tomato hypocotyl segments. Auxin directs the expression of the RSI1 gene independently of the *DGT* gene (Young et al., 1994) whereas the induction of four other genes (*TR8* (Young et al., 1994), *BTAS2, BTAS3* and *LeSAUR*) depends on a functional *DGT* gene. Cytokinin reduces the auxin-inducibility of two of the genes which require *DGT* for their induction (*BTAS2* and *TR8*).

elongation, or the inhibitory action of cytokinin on the elongation response may occur downstream from LeSAUR expression.

Taken together, our data suggest that the effects of cytokinin and the *dgt* mutation on plant development may be similar because cytokinin reduces the amplitude of auxin-induced responses, such as cell elongation and ethylene synthesis. These inhibitory effects of cytokinin appear to be mediated, at least in part, through the inhibition of auxin-induced gene expression which likely occurs downstream from the action of the *DGT* gene product.

III.5 Materials And Methods

III.5.1 Plant Materials

The *dgt* mutant in the background Ailsa Craig (AC) was obtained from Dr. C.M. Rick, U.C. Davis. The mutant gene was extensively back-crossed into AC from the original VFN8-line (C.M. Rick, personal communication). All seeds used in this study came from field plants propagated by selfing at the Oregon State University Botany Farm. Seeds were bleached for 30 minutes in 50 % household bleach, rinsed in tap water, and sown in plastic boxes (32 cm x 26 cm x 10 cm) onto two layers of Whatman filter paper #3 moistened with distilled water. The covered boxes were incubated for 3 to 5 days at 28°C in the dark.

III.5.2 Auxin-induced Elongation

Segments for elongation experiments were harvested and handled under green safelight. Hypocotyl segments 6 mm in length were cut from immediately below the hook of seedlings which were 1 to 2 cm tall. For each treatment, 15 segments were floated on sucrose/MES (SM) buffer (1% sucrose, 5 mM MES/KOH, pH 6.0) for a 2 h pre-incubation period in darkness at 28 °C. At the end of the pre-incubation, segments were measured with a dissecting microscope equipped with an ocular micrometer and transferred to SM buffer containing the appropriate growth regulators. Segments receiving BAP during the incubation period also received BAP during the pre-incubation. After 14 more h of incubation in the light (145 μE $m^{-2}s^{-1}$), the segments were re-measured, and the average length increase was calculated.

III.5.3 Auxin-induced Ethylene Synthesis

Measurements of ethylene synthesis were performed essentially as described by Kelly and Bradford (1986). Hypocotyl segments were harvested and pre-incubated as described for elongation assays. For each treatment, 15 segments one cm in length were floated on 1 ml of SM buffer containing the appropriate growth regulators in a 10-ml vial. Vials were incubated uncapped for 2 h, then sealed and incubated for 3 more h to allow ethylene to accumulate. All incubations were under agitation at 28°C in the dark. One ml of the gas phase of each sample was analyzed on a Shimadzu gas chromatograph equipped with a 4-foot Poropak Q-column and a flame-ionization detector. Afterwards, the

segments were gently blotted on Kimtowels (Fisher, Pittsburgh, PA, USA) and weighed to normalize ethylene production for tissue fresh weight.

III.5.4 Gene Expression

Hypocotyls were excised from 5 day old etiolated seedlings and cut into pieces approximately 1 cm in length. The segments were pre-incubated for 2 h in SM buffer and then incubated for 2 more h in SM buffer containing either 100 µM IAA, 100µM BAP, 70 µM cycloheximide or a combination thereof. Segments treated with BAP during the final incubation period also received 100 µM BAP during pre-incubation. All incubations were done at 28°C in the dark under gentle agitation.

RNA was prepared by extraction in the presence of guanidinium thiocyanate essentially as described in Ausubel et al. (1991). Hypocotyl segments were weighed, frozen in liquid N_2 and stored at -80°C. Tissue was thawed at 37°C in 1.5 ml/g extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5 % sarcosyl, 0.762% β-mercaptoethanol) until the hypocotyl segments separated, and then ground three times for 15 sec. at full speed using a Tekmar homogenizer fitted with a small probe. After grinding, 150 µl of 2 M sodium acetate, pH 4.0 and 1.5 ml water-saturated phenol and 300 µl chloroform:isoamyl alcohol (49:1) were mixed in for each g fresh weight, and the extracts were spun for 20 min at 3600 rpm (3000 x g) in a Beckmann GPR (Beckmann Instruments, Fullerton, CA, USA) tabletop centrifuge. The upper aqueous phase was re-extracted with an equal volume of chloroform:isoamyl

alcohol (49:1) and then precipitated with an equal volume of isopropanol. The RNA pellet was resuspended in diethylpyrocarbonate (depec)-treated water and re-precipitated overnight at 4°C after adding an equal volume of lithium-chloride solution (4 M lithium chloride, 20 mM sodium acetate, pH5.2). The pellets were resuspended in 150 µl depec-treated water and re-precipitated by adding 2.5 µl of 5M sodium chloride and 380 µl nucleic acid grade ice cold ethanol (100 %). Dried pellets were resuspended in depec water, and concentration and purity of the RNA were checked by comparing the absorbance at 320, 260 and 280 nm in a Beckman DU-64 spectrophotometer.

For northern blots, 10 µg of total RNA were denatured by heating for 12 min in 12.3 µl depec water, 0.5 µl formamide, 1.7 µl of 37% formaldehyde, 1 µl of 10 x MOPS and 1 µl ethidium bromide (1 mg/ml). The denatured RNA was electrophoresed on 1.2% agarose gels containing 1.2 M formaldehyde for 3 h at 80 V. Equal loading of the lanes was confirmed by comparing the intensity of ethidium bromide-stained rRNA bands. RNA was transferred to HybondN nylon membranes (Amersham, Arlington Heights, IL, USA) in 10 X SSC by capillary transfer overnight, and crosslinked to the membranes by baking at 80°C for 1 h. Membranes were wetted in 6X SSC, and then prehybridized for at least 3 h at 60 °C in 6X SSC, 0.1 % SDS, 2X Denhardt's reagent and 100 µg/ml sheared salmon sperm DNA. Hybridization was in the same solution at 65°C in a rotary hybridization oven (Robbins Scientific, Model 2000) overnight. Filters were washed twice at room temperature in 2X SSC, 0.1% SDS, and then for 1 h at 55°C and 1X SSC, 0.1% SDS. The *LeSAUR* probe was synthesized by labeling

a 100 base pair XhoI-BamHI fragment of the plasmid described by Mito and Bennett (1995). Labeling reactions were carried out using the Decaprime kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Unincorporated nucleotides were removed by passage of the labeling reaction over a Sephadex G-50 column. 1.5 x 10^5 cpm of probe were added to 5 ml of hybridization solution.

The [^{35}S]-labeled probes for RNase protection assays were generated by linearizing plasmids with BamHI and transcribing those templates from their T7 promoters using the Maxiscript kit (Ambion) according to the manufacturer's instructions. Unincorporated nucleotides were removed as above. RNase protection assays were carried out using the Ambion RPAII kit. The probes (2 x 10^5 cpm) were hybridized with 20 µg of total RNA at 42°C over night in a hybridizing oven (VWR 1540; VWR, Seattle, WA, USA) and unhybridized RNA was digested with a mixture of ribonucleases A and T1 according to the manufacturer's instructions. Radiolabeled fragments were analyzed by native polyacrylamide gel electrophoresis and fluorography.

The signals on films from northern blots and RNase protection assays were quantified by scanning the films in a densitometer (Molecular Dynamics, Sunnydale, CA, USA). The relative signal intensities were expressed as % of the maximum signal obtained in each experiment. Relative signal intensities obtained from three independently grown and treated batches of hypocotyl tissues were averaged and the variability was expressed as the standard error of the means (SE) from the three experiments.

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise.

III.6 Acknowledgements

We are grateful to Dr. Shang Fa Yang and his collaborators for providing the clones for the tomato ACC-synthase genes and to Dr. Alan Bennett and Dr. Nobuaki Mito for use of the *LeSAUR* clone. We thank Dr. David L. Rayle and Dr. Mary J. Ellard-Ivey for valuable discussions and practical advice. This research was supported by NSF grants to T.L.L.

IV. AUXIN AND CYTOKININ INDEPENDENTLY REGULATE ORGAN AND TRACHEARY ELEMENT DIFFERENTIATION AND INTERACTIVELY CONTROL CALLUS GROWTH IN TOMATO (*LYCOPERSICON ESCULENTUM* MILL.)

Catharina Coenen and Terri L. Lomax

Department of Botany and Plant Pathology and Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR 97331-2902

IV.1 Summary

We have used the auxin-insensitive *diageotropica* (*dgt*) mutant of tomato (*Lycopersicon esculentum* Mill.) to investigate the interaction of auxin and cytokinin in the growth and differentiation of plant tissues in culture. Mutant hypocotyl explants regenerated both roots and shoots, however with much reduced vigor compared to wild-type explants. Shifts in the hormone sensitivity of organ regeneration in the mutant suggest that the auxin responses affected by the *dgt* gene modulate organ formation. Production of callus from wild-type hypocotyl explants and growth of transferred wild-type calli showed clear dose responses to both auxin and cytokinin. Mutant explants produced small amounts of callus, demonstrating that the *dgt* mutation does not disrupt callus formation *per se*. However, mutant calli were not stimulated by either auxin or cytokinin in a concentration-dependent fashion. The number of tracheary elements per g of wild-type callus was reduced by the auxin 2,4 dichlorophenoxyacetic acid (2,4-D) and stimulated by the cytokinin 6-benzylaminopurine (BAP). Tracheary element content of *dgt* callus showed reduced responsiveness to 2,4-D while the response to BAP was intact. These characteristics of the *dgt* mutant in tissue culture indicates that the *DGT* gene is required for the concentration-dependent modulation of organ regeneration, callus growth, and tracheary element differentiation by auxin. The effects of cytokinin and the *dgt* mutation on organ formation and vascular differentiation were additive, whereas the stimulation of cell division by cytokinin was dependent on a functional *DGT* gene. Our results

suggest that cytokinin and the auxin response which is mediated by the *DGT* gene product control organ formation and vascular differentiation independently, but that they act through a common signal transduction pathway to stimulate cell division.

IV.2 Introduction

Many growth and differentiation processes during plant development are thought to be regulated by the phytohormones auxin and cytokinin. The two hormones can have opposing effects on the differentiation of plant tissues, such as in the regeneration of shoots or roots in tissue culture (Skoog and Miller, 1957), and in the control of the size and density of vascular elements in plant stems (Aloni, 1995). Cytokinin can act synergistically with auxin, for example to stimulate cell division in cultured tissues (Skoog and Miller, 1957; Das et al., 1956; Bottomley et al., 1963), or as an inhibitor of auxin-induced cell division during the initiation of lateral root primordia in vivo (Torrey 1956, 1962; Bottger, 1974; Wightman et al., 1980; Hinchee and Rost, 1986).

The culture of isolated plant tissues and organs in vitro has been used extensively to study the effects of auxin and cytokinin on plant tissues as well as the interaction between these two hormones (for review, see Krikorian, 1995). Tissue culture can be used to investigate growth and differentiation processes at the whole organ level, such as the regeneration of shoots and roots from tissue

explants or callus, or to study cell division and the differentiation of specialized cell types such as tracheary elements (TEs) in callus or pith explants. Traditionally, the effects of auxin and cytokinin on plant organs and tissues in culture are studied by varying the levels of these two hormones in the growth medium. A requirement for auxin and cytokinin in cell division and in differentiation processes such as tracheary element formation was established by studies on callus in vitro (e.g. Fosket and Torrey, 1969).

More recently, the development of transgenic plants has made it possible to study the effects of varying endogenous levels of auxin and cytokinins. These studies have largely confirmed the observations made with exogenously-applied hormones (reviewed in Hamill, 1993). Transgenic plants have successfully been used to demonstrate that the ratio of auxin to cytokinin regulates apical dominance in whole plants, that auxin and cytokinin have opposing effects on vascular development in vivo, and that root development is inhibited by increasing either auxin or cytokinin levels (reviewed in Klee and Estelle, 1991; Hamill, 1993; Klee, 1994). Few studies have addressed the changes in hormone sensitivity in hormone-over- or underproducing transgenic plants. Plants overproducing cytokinins due to transformation with a bacterial gene encoding isopentenyltransferase (*ipt*) under the control of an auxin-inducible promoter showed increased tolerance to the growth inhibitory effects of exogenously applied auxin (Li et al., 1994). The endogenous synthesis of an auxin antagonist, identified as indole lactate, was found to enhance shoot

formation on media containing auxin concentrations which inhibit shoot formation in wild-type calli (Körber et al., 1991).

A different approach can be taken with more traditional genetic methods using mutants which have altered responses to auxin or cytokinin. While a large number of such mutants are now available (Reid, 1990; Hobbie and Estelle, 1994; Deikman and Ulrich, 1995), the use of these plants to study differentiation and growth in tissue culture has been limited. NAA-resistant mutants of *N. tabacum* were found to be impaired in root morphogenesis and regenerated shoots show darker green and more lanceolate leaves but were otherwise normal in morphology and development (Muller et al., 1985). The *axr1* mutant of *Arabidopsis* shows significantly less callus development than wild-type when inflorescence segments are incubated on media containing 1 to 20 μM 2,4-D (Lincoln and Estelle, 1991). A cytokinin-resistant mutant of *Arabidopsis*, *cyr1*, requires higher concentrations of cytokinins than the wild-type to induce shoot regeneration from root explants (Deikman and Ulrich, 1995). Another *Arabidopsis* mutant, *stp1*, was found to have a reduced response to cytokinin in root elongation assays without affecting cytokinin responses in callus growth, or in the regeneration of shoots from root explants (Baskin et al., 1995).

The *diageotropica* (*dgt*) mutant of tomato (*Lycopersicon esculentum* Mill.) is a well-characterized, single-gene, recessive auxin response mutant. Morphology (Zobel, 1994; chapter II), physiology (Kelly and Bradford, 1987, Muday et al., 1995; chapter III) , and gene expression (Mito and Bennett, 1995;

chapter III) in this mutant are consistent with the idea that the *DGT* gene is required for the responses of tissues to auxin. In this paper we use the *dgt* mutant to explore the relationship between auxin and cytokinin responses in organ regeneration, callus growth and the differentiation of vascular elements. The results demonstrate that cytokinin and the auxin response mediated by the *DGT* gene have additive effects on organ regeneration and tracheary element formation, indicating that cytokinin and auxin act independently on these processes. In contrast to this, the absence of a cytokinin response in *dgt* callus growth suggests that cytokinin acts on a *DGT*-dependent pathway to stimulate cell division.

IV.3 Materials and Methods

IV.3.1 Plant Material

The *dgt* line used in this study was an EMS-induced allele of the *dgt* mutant in the background line VF36. Seeds of *dgt* and its isogenic parent were originally obtained from Dr. R. Zobel, Cornell University, Ithaca, NY. All seeds used in this study came from field plants propagated by selfing at the Oregon State University Botany Farm.

IV.3.2 Tissue Culture

Seeds (0.7 g) were surface-sterilized in 80 ml of 50% household bleach containing 2 drops of Tween 20. The bleach solution was removed by rinsing

the seeds 5 times for two min each in 200 ml of sterile distilled water. Subsequently, 0.15 g of seeds were spread on 4 layers of Whatman #1 filter paper wetted with 9 ml of sterile distilled water in a sterile plastic Petri dish (10 cm diameter, 2.5 cm deep). The dishes were sealed with Parafilm and incubated at 28°C in a light incubator equipped with wide-spectrum fluorescent lights (General Electric, Plant and Aquarium). Seedlings were grown for nine days under a cycle of 16 h light (50 µE of PAR/m^2s) and 8 h dark.

Tissue culture media contained 0.8 % agar, MS-salts and B-vitamins, and the indicated concentrations of hormones. Growth regulator stocks were prepared as aqueous solutions by heating until dissolved. Hormones and vitamins were added before autoclaving the media. Fifty ml of media were poured in sterile plastic Petri dishes (10 cm diameter, 2.5 cm deep) and left to cool and dry at room temperature over night.

Four hypocotyl explants of approximately 1 cm length were cut from immediately below the cotyledons of 9-day old seedlings and were placed on each culture dish. For experiments on organ formation, dishes were incubated under the same conditions as seedlings were grown (see above). Photographs were taken after one month. For callus induction, dishes with explants were incubated for one month at 28 °C in the dark. To characterize growth of transferred callus, explants were placed on plates containing 3 µM 2,4-D and 3 µM BA and incubated in the dark (28 °C) for one month, callus was then cut away from the original explants and callus pieces (0.5 cm x 0.5 cm x 0.5 cm)

were transferred to fresh plates containing the same growth regulator concentrations (four pieces per plate). After one month, the callus was cut into pieces as before and transferred to fresh plates containing hormone combinations as indicated.

IV.3.3 Cell Counts

Callus pieces were weighed and digested at room temperature in 5% (w/v) chromium trioxide, 5% (v/v) HCl (modified after Fosket and Torrey, 1969). After 24 h, the tissue was macerated by passing it five times through an 18 G hypodermic needle. The macerates were spun for five min. at 1000 rpm (225 x g) in a Beckman GPR tabletop centrifuge. Supernatants were removed and the cell pellet was washed six times by resuspending in double distilled water and spinning at 225 x g. For the last wash, the water was replaced by 1 mM Tris, adjusted to pH 7.5 with HCl. The final supernatants showed no yellow discoloration and had a neutral pH. The cells were spun one more time and the supernatants discarded. The number of cells showing secondary wall thickenings (vascular elements) contained in 0.9 µl of suspension were counted in an improved Neubauer chamber and the results were normalized for the fresh weight of the callus piece. Prior to counting, each cell suspension was adjusted to a final volume which would yield approximately 100 cells in the 0.9 µl chamber.

IV.3.4 Scanning Electron Microscopy

Callus was induced and transfered twice on media containing 3 µM 2,4-D and 3 µM BAP as described above. Callus pieces were cut in half using razor blades and fixed by vacuum infiltration (10 min at 30˝Hg vacuum) and subsequent incubation (3 h at 4 °C) in 2.5% EM grade glutaraldehyde (Electron Microscopy Sciences, Cat. # 16120) in 0.125M cacodylate buffer (pH7.2). Fixed samples were moved through 50, 70, and 100% water/acetone, and then 50, 70, and 100% acetone/trichlorotrifluroethane (TF) solutions (20 min per change). From 100% TF, samples were critical point dried in a Balzer CPD-020 dryer. Specimens were mounted on aluminum planchettes and sputter coated with 20 nm of 60/40 wt % gold/palladium in an Edwards S150B sputter coater. Specimens were viewed and photographed using an AmRAY 1000A SEM, and images were recorded on Polaroid Type 55 P/N positive/negative 4x5" format film.

IV.4 Results

IV.4.1 Organ regeneration from hypocotyl segments

Cultured wild-type hypocotyl explants regenerated vigorous and prolific roots and shoots in the absence of hormones (Fig. IV.1a, plant on the left). Hypocotyl explants of *dgt* were also able to regenerate both leaves and roots, however these organs were much less prolific and vigorous than those

Fig. IV.1 Low levels of auxin or cytokinin differentially modulate organ regeneration in wild-type and *dgt* hypocotyl explants. **(a.)** Regeneration of leaves and roots from untreated wild-type and mutant hypocotyl segments. **(b.)** Enhancement of organ formation from *dgt* hypocotyl explants in the presence of 0.3 µM 2,4-D. **(c).** Phenocopy of *dgt* development in wild-type explants by treatment with 0.3 µM BA.

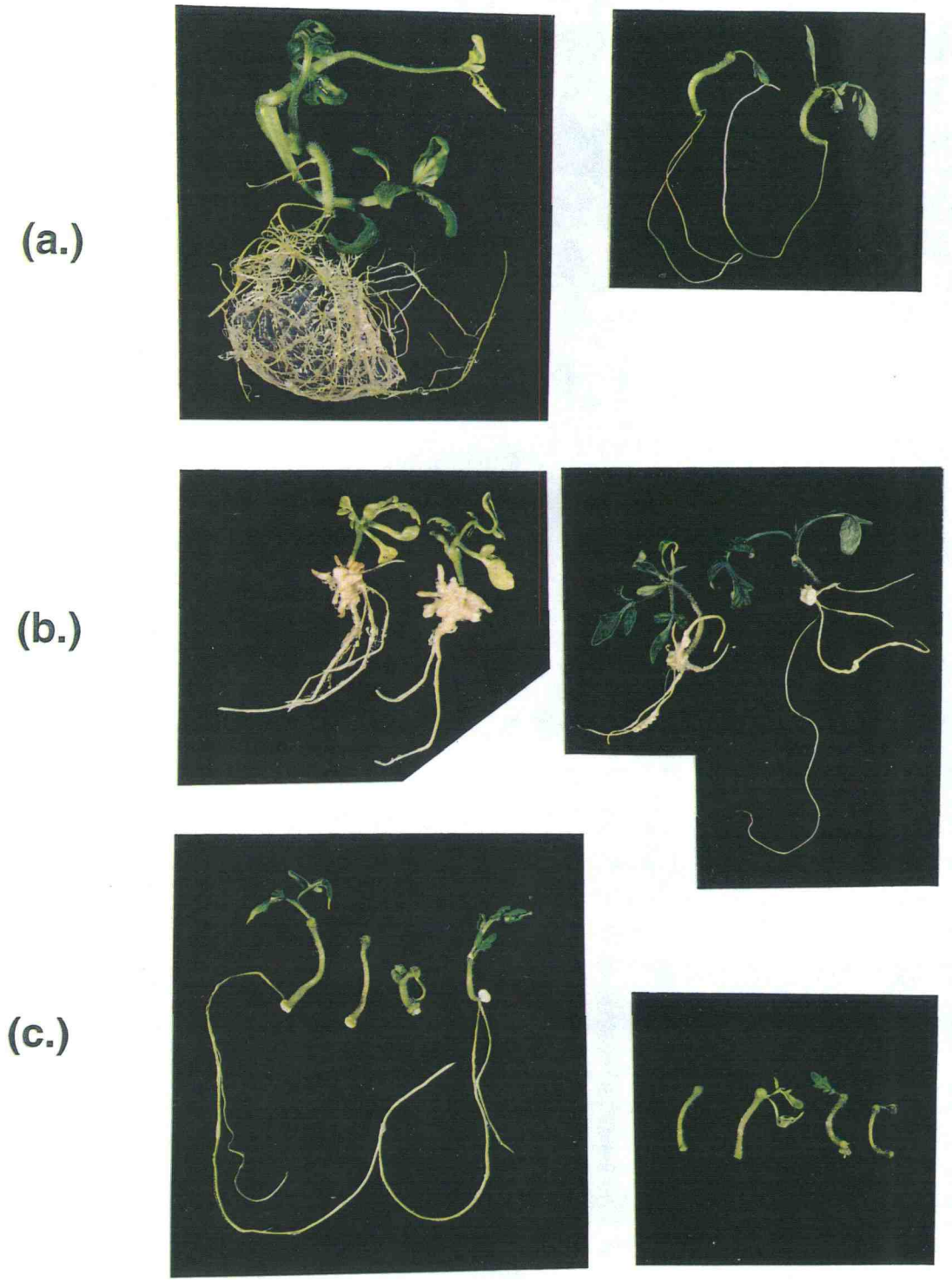

Figure IV.1

regenerated from wild-type explants (Fig. IV.1a, two plants on the right). Similar to intact *dgt* plants, roots regenerated by *dgt* hypocotyl explants did not form lateral roots. In both genotypes, leaves emerged from the apical end of the explants and roots from the basal end, indicating that the polarity of the hypocotyl segment was retained.

Morphogenesis in mutant and wild-type explants differed with respect to the effects of low auxin concentrations. For example, wild-type leaves produced in the presence of 0.3 μM 2,4-D were smaller and much paler than those produced by untreated wild-type explants (compare plants on the left of Fig. IV.1a and IV.1b), while *dgt* leaves regenerated in the presence of 0.3 μM 2,4-D were as green, and more vigorous, and numerous than *dgt* control leaves (plants on the right of Fig. IV.1a and IV.1b). Auxin treatment thus partially rescued the *dgt* phenotype with respect to leaf number and vigor, although it did not restore root branching in *dgt*.

Cytokinin treatment resulted in a phenocopy of *dgt* development in wild-type explants. In the presence of 0.3 μM BA, wild-type explants produced much weaker and smaller roots and leaves than the control explants, and root branching was nearly absent (Fig. IV.1c), so that these plants resembled the *dgt* controls (compare plants on the left of Fig. IV.1c with plants on the right of Fig. IV.1a). Mutant explants treated with 0.3 μM BA did not produce any roots and the regenerated leaves were smaller than those of the controls, indicating that

the effects of cytokinin and the *dgt* mutation on the regeneration of roots and shoots may be additive.

Whereas differences in organ regeneration between wild-type and *dgt* explants were apparent at low hormone concentrations, the overall pattern of organ differentiation in response to various concentrations of either hormone in mutant and wild-type explants was very similar (Fig. IV.2). At the higher cytokinin concentrations (3 and 30 µM), explants from *dgt* and wild-type looked almost identical, irrespective of the auxin concentration used. A subtle difference between *dgt* and wild-type explants occured when high auxin concentrations were used in combination with the high cytokinin. Under these conditions *dgt* explants produced slightly more callus and remained greener. Root induction in mutant calli was less effectively suppressed by auxin than in wild-type calli. At 3 µM 2,4-D, mutant calli formed numerous roots, while this concentration of 2,4-D suppressed root formation in wild-type almost completely. In addition, *dgt* calli formed roots at low equimolar concentrations of 2.4-D and BA (0.3 µM), whereas wild-type calli did not (Fig. IV.2). Another subtle difference was that callus formed by wild-type explants tended to be browner than mutant callus when high auxin concentrations (30 or 100 µM) were applied.

IV.4.2 Induction and Growth of Callus

In order to suppress organ formation as much as possible in experiments testing the effect of the *dgt* mutation on callus growth, explants for callus induction were incubated in the dark. Optimal callus induction in both genotypes

Fig. IV.2 Hormone matrix demonstrating the effects of BAP and 2,4-D on callus production and regeneration of organs from tomato hypocotyl segments. For each combination of hormone concentrations, the wild-type explant is on the left and the *dgt* explant is on the right.

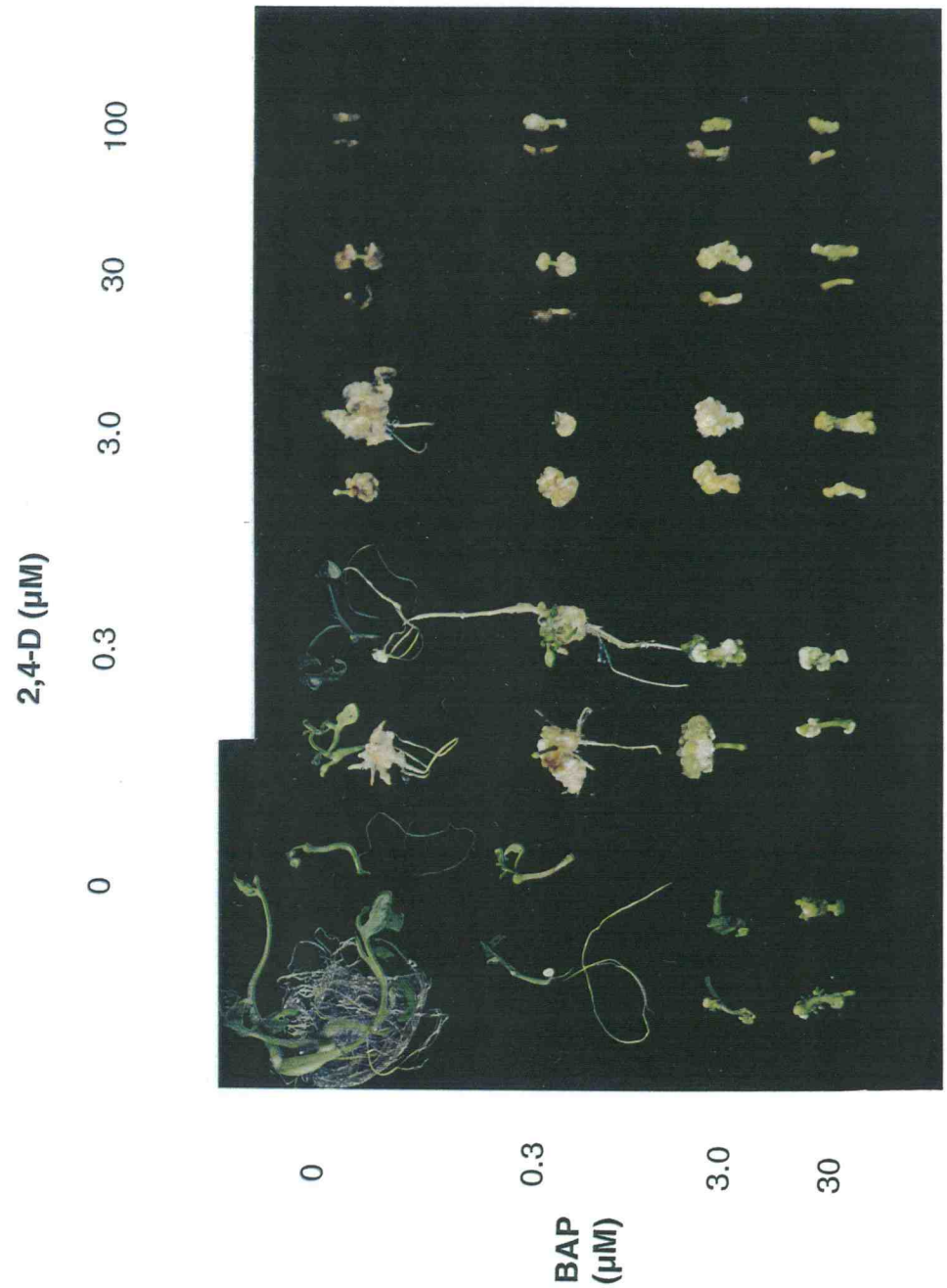

Figure IV.2

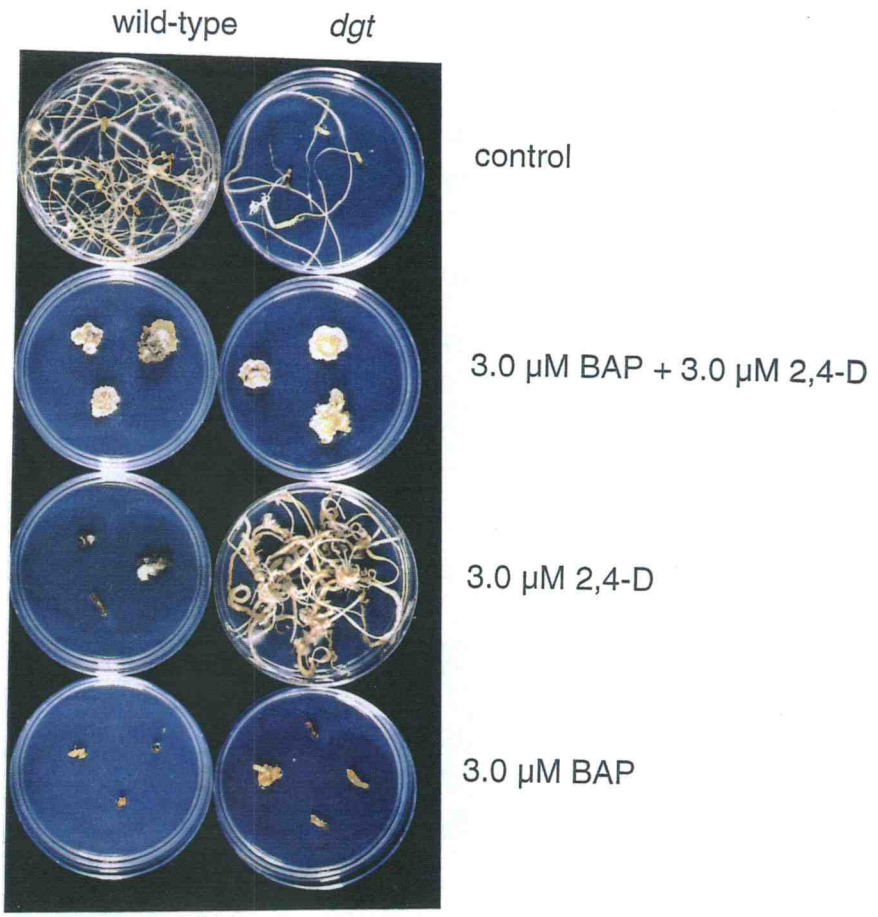

Fig. IV.3 Callus induction in wild-type and *dgt* explants.

was dependent on the presence of both auxin and cytokinin (Fig. IV.3). With no growth regulators added, hypocotyl explants from both wild-type and *dgt* produced small amounts of callus and roots. Roots formed from untreated explants were more prolific in wild-type explants than in *dgt* explants. Addition of 3 μM 2,4-D to the medium stimulated callus formation and suppressed root formation in wild-type explants but this auxin concentration stimulated rather than inhibited root formation in the mutant. Cytokinin suppressed root formation in both genotypes.

When the production of callus by hypocotyl explants was studied on a hormone matrix (Fig. 4a), callus production from wild-type hypocotyl explants was most prolific in the presence of 10 μM BA and 1 μM 2,4-D. Fresh weight of wild-type calli showed clearly defined dose responses to both auxin and cytokinin. In contrast, mutant hypocotyl explants produced approximately equal amounts of callus tissue in response to all growth regulator combinations tested in this experiment, showing no increase in response to cytokinin or auxin (Fig. IV.4a).

To test whether the lack of hormone responsiveness in mutant tissue was dependent on the presence of the original explant, callus was induced on a medium containing 3 μM BA and 3 μM 2,4-D, excised away from the original explant, subcultured once on the same medium, and then transferred to media containing various combinations of BA and 2,4-D. The callus growth response in both wild-type and mutant tissues after two passages was similar to callus

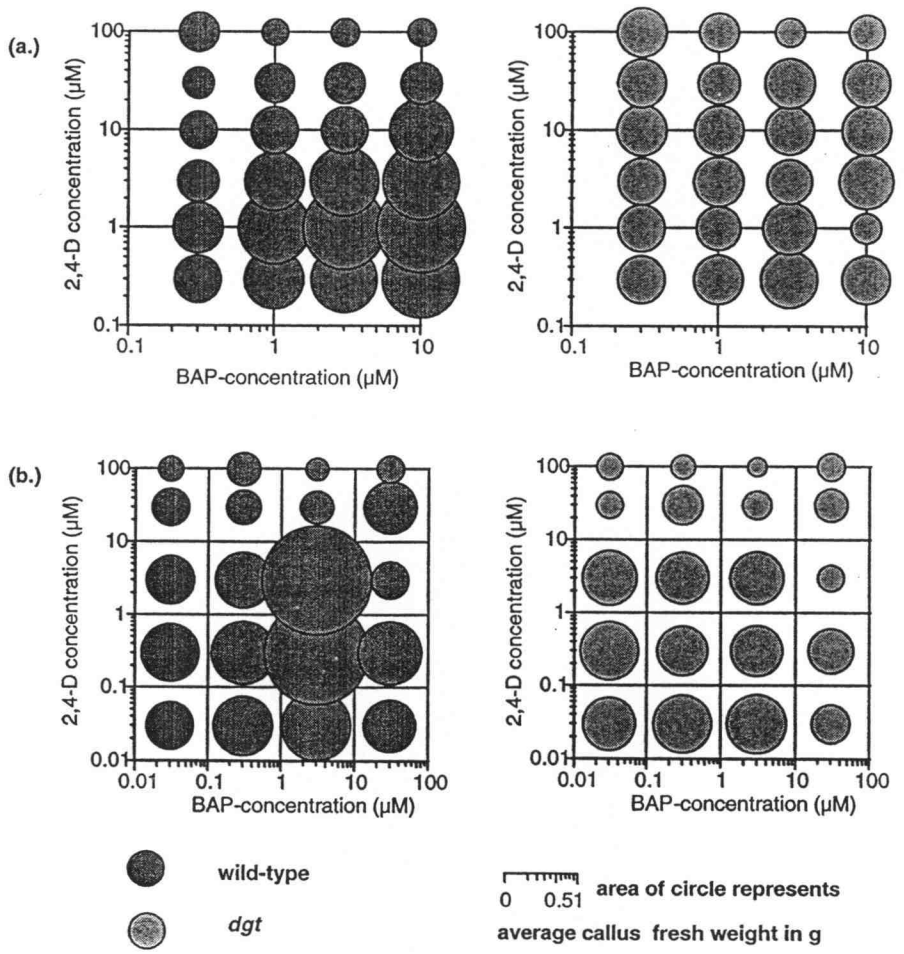

Fig. IV.4 Responses of callus induction and growth to 2,4-D and BAP in wild-type and *dgt*. **(a).** callus induction from hypocotyl segments in the dark. **(b).** growth of subcultured callus in the light. Each data point represents the average fresh weight of 4 callus pieces.

induction from hypocotyl explants (compare Fig. IV.4a and IV.4.b). The growth response of wild-type callus showed clear optima for both auxin and cyotkinin concentrations, whereas there were no such optima for *dgt* callus growth (Fig. IV.4b).

Mutant callus morphology differed from wild-type in that mutant calli remained hard and white irrespective of the hormone treatment, whereas wild-type calli grown at near-optimal hormone concentrations were soft and translucent. Mutant calli which had been passaged twice on medium containing 3 µM 2,4-D and 3 µM BAP contained more vascular elements than wild-type calli (examples shown in Figs. IV.5 and IV.6). In wild-type calli grown in the presence of 2,4-D alone, the number of vascular elements per g of callus fresh weight was reduced to about half of the number found in calli grown in the absence of hormones. In contrast, BA induced a doubling in the number of vascular elements. In the presence of both hormones, the number of vascular elements per g of callus fresh weight was the same as in untreated control tissue. In *dgt* callus, the reduction in vascular element density by auxin-treatment was not as pronounced as in wild-type. BA caused a doubling of the number of vascular elements relative to control callus, so that the cytokinin response of *dgt* calli with respect to vascular differentiation appeared normal. In contast to the response in wild-type, auxin failed to reduce the number of vascular elements back to control values in the presence of BA. Thus the lack of an auxin-induced

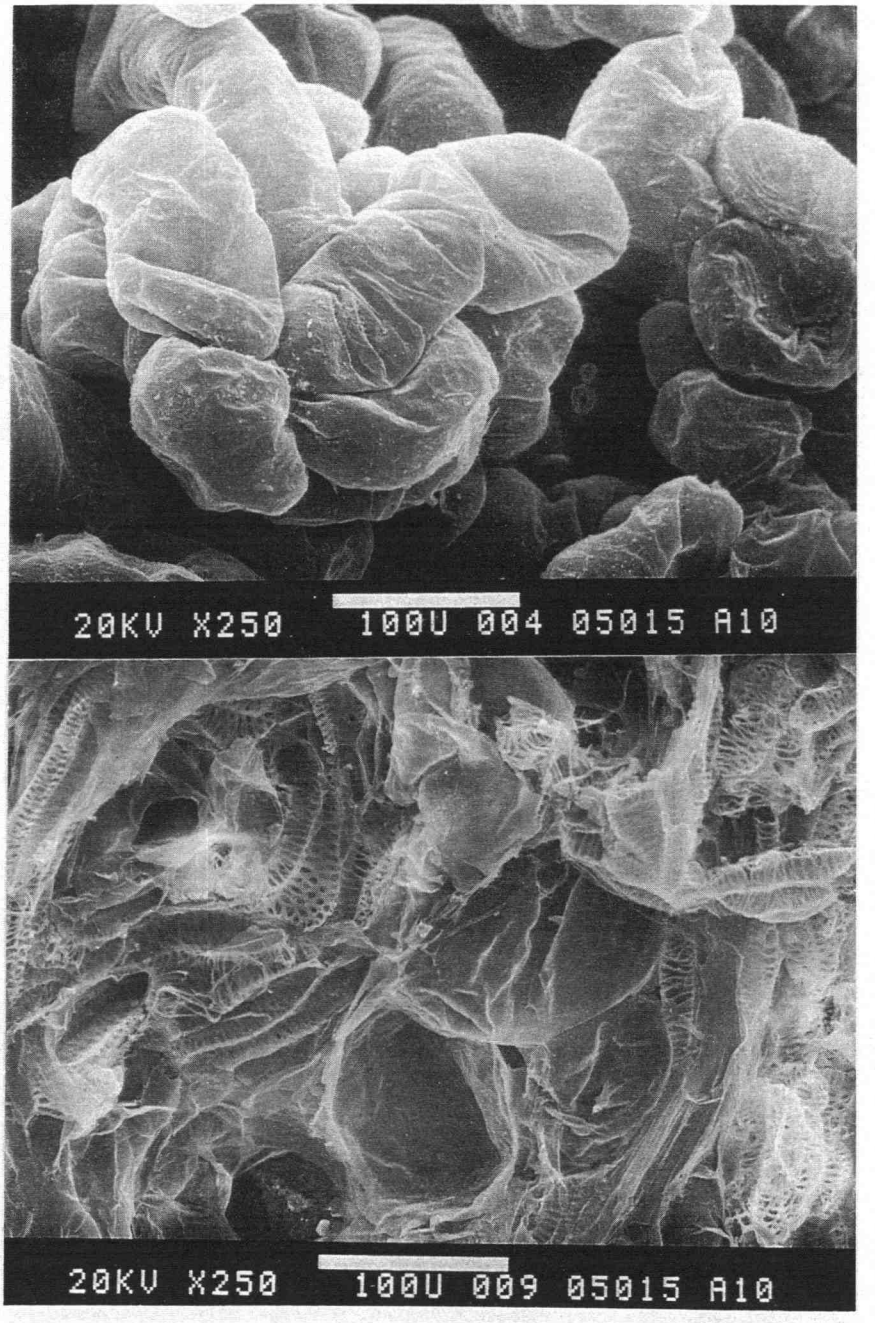

Fig. IV.5 Representative scanning electron micrographs of cells in wild-type (top) and *dgt* (bottom) callus after two passages on media containing 3 μM 2,4-D and 3 μM BAP. Note the clusters of tracheary elements in the *dgt* callus.

suppression of vascular differentiation may be in part responsible for the altered morphology of *dgt* calli.

IV.5 Discussion

IV.5.1 Effects of the *dgt* Mutation on Growth and Differentiation *in vitro*

Traditional physiological experiments (e.g. Skoog and Miller, 1957; Fosket and Torrey, 1969) and studies with auxin-auxotrophic cell lines (Blonstein et al, 1988; Fracheboud and King, 1991; Oetiker et al., 1990) demonstrate that auxin is essential for the regeneration of leaves and roots and induction of callus. Although hypocotyl segments of the *dgt* mutant were previously shown to be auxin-insensitive (Kelly and Bradford, 1986), *dgt* hypocotyl segments regenerated both leaves and roots and can also be induced to form callus, which demonstrates that the *dgt* mutation does not eliminate these auxin-dependent developmental programs (Figs. IV.1 and IV.2). There are two possible interpretations for our observations on the *dgt* mutant. Either the *dgt* mutation is leaky or the *DGT*-dependent response pathway is not essential for the elicitation of these developmental programs.

The lack of auxin responsiveness in *dgt* hypocotyl segments is close to complete (Kelly and Bradford, 1987; see also chapter III), which argues against a leaky *dgt* mutation. Furthermore, the resistance of *dgt* roots to auxin in root growth inhibition assays (Muday et al., 1995) is comparable to the level of

resistance observed in the strongest alleles of auxin-insensitive *Arabidopsis* mutants (compare with Timpte et al., 1995).

Alternatively, the *DGT* gene product may not be required for the elicitation of these developmental programs. Rather, *DGT* may operate in a separate pathway mediating the sensitivity of these responses to changing auxin concentrations. This interpretation would explain why *dgt* hypocotyls produce a certain amount of callus in response to auxin (Fig. IV.3), but do not show the typical auxin response curves for callus growth which are seen in wild-type callus (Fig. IV.4).

The existence of at least two separate but interacting auxin response pathways has already been proposed for *Arabidopsis* (Timpte et al., 1995). This proposal was based on the finding that two auxin-resistant mutants, *aux1* and *axr1*, are additive in their effects on the auxin response. The existence of a second, *DGT*-independent, auxin-response pathway in tomato is supported by studies which demonstrate that auxin increases transcript levels for two auxin-regulated genes in *dgt* tissues (Mito and Bennett, 1995; Young et al, 1994). One of these genes, *LePAR*, has high sequence similarity to the tobacco gene *parA* (Mito and Bennett, 1995), which is expressed in tobacco mesophyll protoplasts during the transition from the G_0 to S phase, and thus may be involved in cell division (Takahashi et al., 1989). The other gene which has been found to be auxin-inducible in *dgt* tissues, *RSI1*, is thought to play a role in the cell divisions initiating lateral root formation (Young et al., 1994). These two

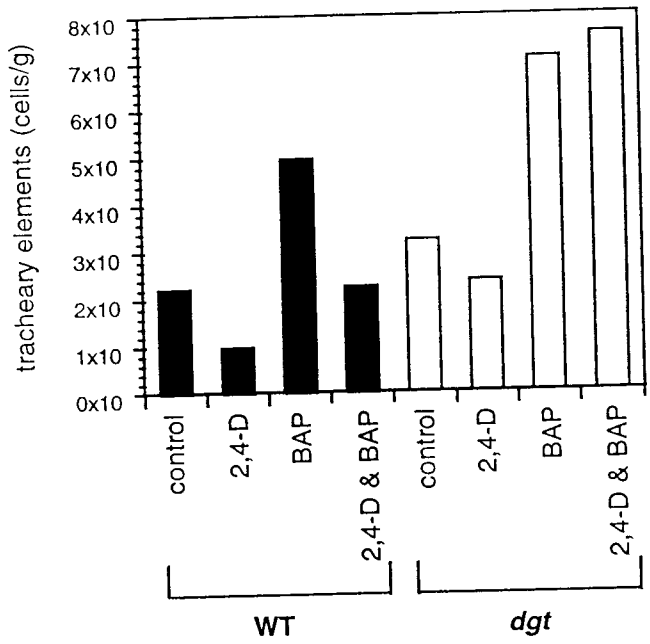

Fig. IV.6 Hormone effects on vascular element content of wild-type and *dgt* callus.

genes may therefore be in an auxin-induced, *DGT*-independent pathway leading to cell division.

Auxin treatment reduced the density of tracheary elements in wild-type callus (Fig. IV.6). These data are preliminary, and repetition and replication of this type of experiment are required. However, the effect of 2,4-D on TE density in the presence or absence of BAP were similar. Suppression of TE differentiation by 10 μM 2,4-D has previously been reported for soybean callus (Fosket and Torrey, 1969). However, the 2,4-D-induced reduction of the total number of TEs formed in the soybean callus was closely associated with a reduction in total cell numbers. In contrast to these observations, 2,4-D concentrations used in our experiment (3 μM) stimulated rather than inhibited callus growth (Fig. IV.4), suggesting that 2,4-D either suppresses TE differentiation or that it selectively enhances the proliferation of cells which do not differentiate into TEs. The negative effect of 2,4-D on the number of TEs in wild-type callus agrees well with the observation that calli formed by the auxin-response mutant *dgt* contain elevated numbers of TEs (Fig. IV.5). Together, these data suggest that the *DGT* gene may function in an auxin-induced signal transduction pathway which either inhibits the differentiation of TEs or selectively stimulates the growth of cells which do not differentiate into TEs.

IV.5.2 Cytokinin Interactions with *DGT*-dependent Auxin Responses

The interaction between auxin and cytokinin in the control of growth and differentiation processes has been described in many experimental systems. However, the mechanisms by which these two hormones interact are still unclear. We have used the auxin-insensitive *dgt* mutant to address the question whether the cytokinin responses of tissues *in vitro* are dependent on the presence of a functional auxin response.

The three response pathways presented in Figure IV.7 summarize the three types of interactions between auxin, cytokinin, and the *DGT* gene we observed: (1) a stimulation of the response by the *DGT*-mediated pathway and an independent inhibition by cytokinin, as found for organ formation; (2) a stimulation of the response by cytokinin and an independent inhibition by the *DGT*-mediated pathway, as observed in TE differentiation; and (3) an interacting pathway in which some of the response intermediates for the *DGT*-mediated auxin response and the cytokinin response are shared. All three models propose two separate auxin response pathways, one which elicits auxin responses independently from the *DGT* gene, and a second pathway which is dependent on a functional *DGT* gene and acts to modulate the auxin responses elicited by the first pathway in a concentration-dependent manner.

Because the effects of the *dgt* mutation and cytokinin on tissue regeneration (Fig. IV.1) are additive rather than epistatic, cytokinin and the *DGT*-

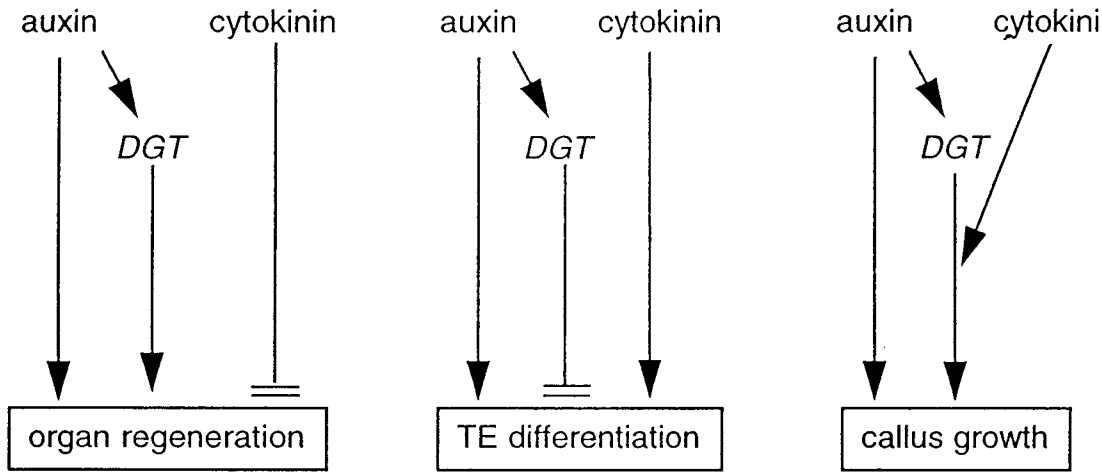

Fig. IV.7 Models for the interaction of auxin, cytokinin, and the *DGT* gene in the control of organ regeneration, tracheary element differentiation, and cell division.

mediated auxin-response likely influence these developmental processes by separate response mechanisms. Similarly, separate response mechanisms for auxin and cytokinin have been proposed for the induction of ethylene synthesis in whole seedlings, because the *dgt* mutation selectively disrupts auxin-induced ethylene formation (Zobel, 1974), while cytokinin-induced ethylene formation is intact in the *dgt* mutant (see Chapter II). Separate auxin and cytokinin response mechanisms may also control TE differentiation (Fig. IV.5), because cytokinins enhances differentiation of TEs in the *dgt* mutant while auxin-reduction of TE densities is impaired.

In contrast, the lack of an auxin-response in *dgt* tissues with respect to callus growth is coupled with the lack of cytokinin-responsiveness (Fig. IV.4). This observation may reflect that auxin and cytokinin stimulate cell division through a common, *DGT*-mediated, response pathway. The idea that the stimulation of cell division by cytokinin is dependent on a functional auxin-response agrees well with studies on a $p34^{cdc2}$-related kinase in tobacco pith (John et al, 1993b). In tobacco pith, auxin induces the synthesis of $p34^{cdc2}$-like protein, and cytokinin is subsequently required to activate this protein. If the *dgt* mutation were to disrupt the auxin-induced expression of such a protein in tomato callus, the mutant would be expected to show no cytokinin-stimulation of callus growth (Fig. IV.7).

Traditional physiological studies have demonstrated that there is more than one mode of interaction between auxin and cytokinin because the two

hormones can have antagonistic, synergistic, or additive effects. Taken together, our results confirm that auxin and cytokinin response pathways interact through multiple mechanisms. Our studies using the *dgt* mutant suggest that auxin and cytokinin can act on a common set of responses either through separate pathways, or through a joint pathway, in which both auxin and cytokinin responses depend on a common signalling intermediate such as the *DGT* gene product.

IV.6 Acknowledgments

We thank Karen Cardozo for the photograph presented in Figure IV.3, and the electron microscopy class of Alfred Soeldner for the electron micrographs in Figure IV.5. The advice of Dr. Donald Armstrong regarding tissue culture techniques and evaluation is greatly appreciated.

V. SUMMARY AND CONCLUSIONS

The interactions between the plant hormones auxin and cytokinin are complex. Qualitative differences in the interaction mechanisms between auxin and cytokinin in different physiological systems are well established through classical physiological studies, demonstrating that cytokinin and auxin can have opposite (Skoog and Miller, 1957; Aloni, 1995; Torrey 1956, 1962; Bottger, 1974; Wightman et al., 1980; Hinchee and Rost, 1986) or synergistic (Skoog and Miller, 1957; Das et al., 1956; Bottomley et al., 1963; Lau and Yang, 1973) effects. However, these studies have generally not yielded information about the mechanism through which the interaction between auxin and cytokinin takes place in these different systems. Recently, combinations of genetic, molecular, and physiological approaches have begun to yield insight into the interactions between different classes of plant hormones (Cary et al., 1995; Su and Howell, 1995; Lehman et al., 1996).

In this study, the *diageotropica* (*dgt*) mutant of tomato has been used to test three general models linking auxin and cytokinin action (Fig. V.1). The first two models propose a linear chain of action in which either auxin controls cytokinin abundance or perception, or vice versa. According to the third model, the interaction between auxin and cytokinin occurs downstream from the perception of either hormone in the response pathway. In all three models, it is assumed that the *DGT* gene acts as a transducer of the auxin signal, as

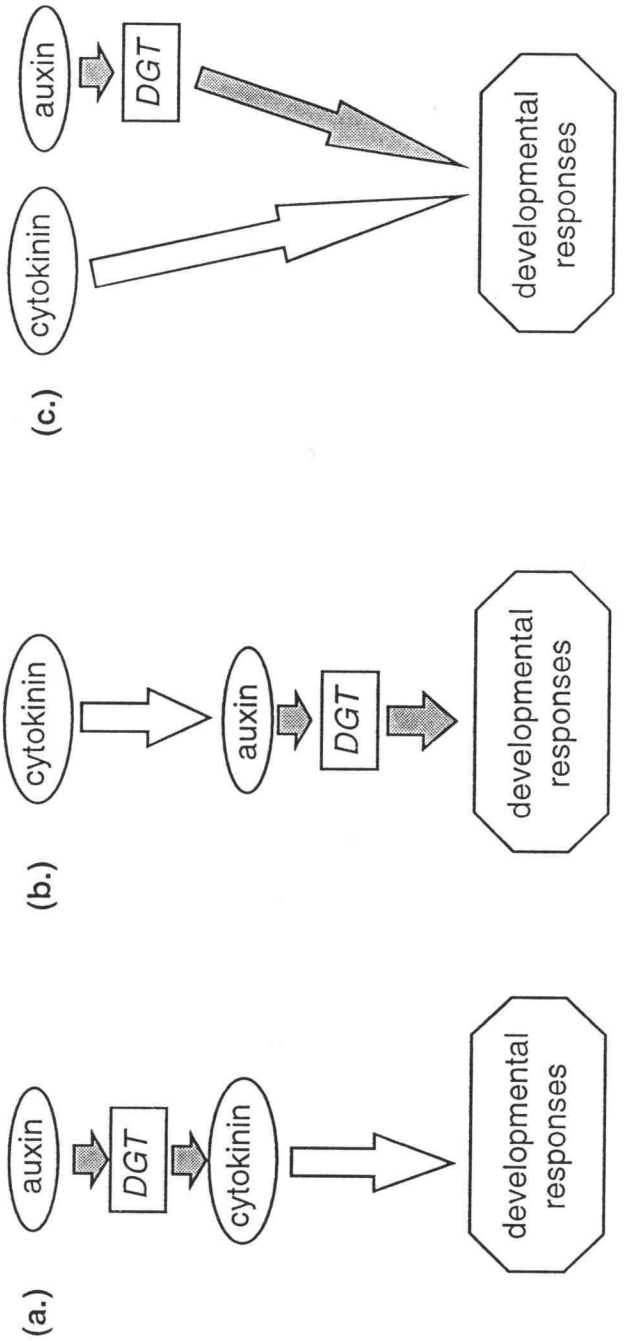

Fig.V.1 General models for auxin-cytokinin interaction.

proposed by Kelly and Bradford (1987), Muday et al., (1994), Mito and Bennett (1995), and Abel and Theologis (1996).

For a system in which auxin and cytokinin have opposite effects, model (a.) predicts that auxin should reduce either the endogenous cytokinin levels or the cytokinin sensitivity of wild-type tissues. According to this model, the *dgt* mutant should be either a cytokinin overproducer or hypersensitive to cytokinin. Model (b.) predicts that cytokinin should reduce the auxin levels or auxin sensitivity in both, mutant and wild-type plants. In addition, the *dgt* mutant should be cytokinin insensitive. Model (c.) assumes a *DGT*-independent pathway for the transduction of the cytokinin signal, and thus predicts that the *dgt* mutation has no effect on cytokinin levels or sensitivity. According to this model, the effects of cytokinin and the *dgt* mutation on developmental responses should be additive.

Initially, the auxin-insensitive *dgt* mutant was used to compare the developmental responses which are controlled by the *DGT* gene with the effects elicited by cytokinin treatment (chapter II). The strong similarities between untreated *dgt* plants and cytokinin-treated wild-type plants showed that the effects of the *dgt* mutation and cytokinin greatly overlap in the range of morphogenetic processes they control, and that they generally act in the same direction. To test whether this similarity could be explained by effects of the *dgt*-mutation on cytokinin metabolism or -sensitivity, endogenous cytokinin levels and cytokinin sensitivity were investigated in etiolated and green seedlings of *dgt* (chapter II). The finding that *dgt* tissues did not show elevated cytokinin levels or

sensitivity as compared to wild-type tissues suggested that the auxin-response which is mediated by the *DGT* gene is unlikely to exert its morphological effects through controlling cytokinin metabolism or sensitivity as shown in model (a.). Rather, the effects of cytokinin and the *dgt* mutation on most responses were additive, thus favoring the view that auxin and cytokinin act through separate response pathways as shown in model (c.). Model (c.) was also supported by experiments demonstrating that *dgt* seedlings were not impaired in their ability to synthesize ethylene in response to cytokinin (chapter II), although *dgt* plants do not produce ethylene in response to auxin (Zobel, 1974),

A second possible explanation for the ability of cytokinin to phenocopy morphological traits of the *dgt* mutant was that cytokinin might act through decreasing the responsiveness of plant tissues to auxin (model (b.)). Physiological experiments on the auxin-induced elongation and ethylene formation in hypocotyl segments demonstrated that treatment with cytokinin did indeed reduce the auxin responses of wild-type tissues (chapter III). However, it was found that cytokinin reduced the amplitude of these auxin responses rather than affecting the sensitivity of the tissues to auxin, suggesting that the molecular mechanisms through which cytokinin and the *dgt* mutation inhibit auxin responses are different. A difference between *dgt*- and cytokinin-induced inhibition of auxin responses also became apparent in experiments on the expression of three auxin-inducible genes. These experiments demonstrated that the effects of the *dgt* mutation were more pleiotropic than the effects of cytokinin treatment, because cytokinin inhibited only one of out of the three

auxin-responsive genes whose expression was affected by the *dgt* mutation. The simplest explanation for these observations is that cytokinin does not act upstream from the *DGT* gene, as shown in model (b.), but rather that it affects gene expression downstream from the *DGT* gene, thus favoring model (c.).

Experiments using a tissue culture system to characterize the interaction between auxin and cytokinin also showed additive effects of cytokinin and the *dgt* mutation on the regeneration of organs and the differentiation of tracheary elements in callus, supporting model (c.) (chapter IV). The traditional model for a synergistic interaction between auxin and cytokinin is the stimulation of callus growth by these two hormones (Skoog and Miller, 1957). Experiments on the growth of tomato callus *in vitro* showed that the cytokinin response in this system likely depends on a functional, *DGT*-mediated response to auxin (chapter IV). A possible explanation for the lack of growth stimulation in *dgt* callus by either auxin or cytokinin may be found in studies by John et al. (1993). Their studies on tobacco pith explants demonstrated an interaction of auxin and cytokinin downstream from auxin-induced gene expression, in which cytokinin activates a $p34^{cdc2}$-like kinase after auxin has increased $p34^{cdc2}$-transcript levels as well as the levels of this protein itself. If the *DGT* gene is required for an auxin-induced increase of transcript levels for a $p34^{cdc2}$-like protein in tomato, *dgt* tissues would lack a target protein for cytokinin to act on.

In summary, the data presented in this thesis best support a model in which auxin and cytokinin responses are mediated by separate pathways which interact downstream from the *DGT* gene product (Fig. V.1.c). However, there is

likely more than one mechanism for this downstream interaction. For example, the gene expression studies described in chapter III demonstrate that one point of interaction is likely upstream from the expression of the ACC Synthase gene *BTAS2*. Suppression of auxin-induced gene expression by cytokinin has also been found in other systems (Van der Zaal et al., 1987; Young et al., 1994), suggesting that this may be a general mechanism for auxin-cytokinin interaction in responses where the two hormones have opposite effects. Stimulatory effects of cytokinin on auxin-induced responses could also be mediated at this level, as cytokinins were shown to enhance auxin-induced gene expression in tobacco suspension cells (Dominov et al. 1992). However, the experiments of John et al. (1993) discussed above show that auxin-cytokinin interactions may also occur downstream from gene expression, suggesting that there are several points of interaction between two separately initiated response pathways.

The models in Figure V.1 are simplified in that they show a single response pathway for auxin. The existence of at least two separate pathways for auxin-signal transduction is indicated by the finding that the auxin resistant *aux1* and *axr1* mutants in *Arabidopsis* act in separate pathways (Timpte et al., 1994) and by gene expression studies showing that certain auxin-inducible genes respond to auxin-treatment in *dgt* tissues (Mito and Bennett, 1995; Young et al., 1994). Furthermore, the survival of auxin-resistant mutants like *dgt* suggests that either all alleles of these mutants isolated so far are leaky, or that the mutant genes act in only one of several auxin response pathways. The close resemblance between *dgt* plants and cytokinin-treated wild-type plants opens the

question whether cytokinin exclusively interacts with *DGT*-mediated auxin-responses. This possibility could be rejected by showing that cytokinin suppresses the auxin-induction of genes which are auxin-inducible in *dgt* tissues. Presently such genes have not been found, however this question merits further investigation.

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